THE SHIKIMATE PATHWAY IN *NEISSERIA GONORRHOEAE*

By

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To the memories, dreams and aspirations of my mother and father.

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THE SHIKIMATE PATHWAY IN *NEISSERIA GONORRHOEAE*

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Neisseria gonorrhoeae was shown to possess a dissociable complex comprising of a phenylalanine-inhibitable isozyme of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DS-Phe) and shikimate dehydrogenase (SDH). Both of the enzymes were active as monomers. DS-Phe activity was found to be partitioned between associated ($DS\text{-}Phe^L$) and dissociated ($DS\text{-}Phe^T$) forms. A M_r of ~ 35,000 was estimated for the monomeric form of DS-Phe by gel-filtration. DS-Phe was found as a dimer (~70,000 daltons) in the presence of one of its substrates, phosphoenolpyruvate (0.75 mM). A M_r of ~ 27,000 was estimated for the monomeric form of SDH by gel-filtration and polyacrylamide gel-electrophoresis. Gel-filtration analysis of crude extracts showed that SDH and DS-Phe existed as components of a larger unit of M_r between 150,000 and 200,000. "Natural" aromatic amino acid auxotrophs of *N. gonorrhoeae* were found to be lacking the activity of shikimate

dehydrogenase. These isolates exhibited pleiotropic loss of both shikimate dehydrogenase and DS-Phe^{L}. An apparent minor and unregulated species of DAHP synthase separated by hydroxylapatite chromatography was shown to be in fact 3-deoxy-D-*manno*-octulosonate 8-phosphate (KDOP) synthase. KDOP synthase was found to utilize erythrose 4-phosphate and ribose 5-phosphate as alternative substrates in place of its normal substrate, arabinose-5-phosphate. However, based on periodate-oxidation studies, the product formed with erythrose-4-phosphate as substrate is suggested to be 3-deoxy-D-*ribo*-heptulosonate 7-phosphate, an isomer of the DAHP synthase reaction product.

CHAPTER 1
INTRODUCTION

The pathway for the biosynthesis of aromatic amino acids is a major metabolic system having many branches. Besides the amino acid end products of the pathway - phenylalanine, tyrosine and tryptophan - a number of other aromatic compounds are synthesized. In bacteria, some of these aromatic compounds are structural units of complex molecules which are vital in their function (see Bentley, 1990, for a comprehensive review). For instance, chorismic acid is not only the last common precursor in the synthesis of the aromatic amino acids, but also of 4-aminobenzoic acid and 4-hydroxybenzoic acid. The latter compounds are precursors of folic acid and ubiquinone, respectively (Bentley, 1990). Isochorismic acid is, likewise, the branchpoint for the syntheses of other important aromatic compounds. Salicylic acid and 2,3-dihydroxybenzoic acid are derived from isochorismic acid and are precursors to siderophores.

The aromatic biosynthetic pathway is present in prokaryotes, plants, and lower eukaryotes (like yeast and fungi). It is absent in man and other animals. Thus, aromatic biosynthesis may be crucial in bacteria that colonize the human host. Since compounds like 4-hydroxybenzoic acid

and 4-aminobenzoic acid are not available from the host, the proliferation of these bacteria may depend on the presence of a functional aromatic biosynthetic pathway. Furthermore, the aromatic pathway offers a highly selective target for anti-metabolite attack that may be pursued with few side-effects on human metabolism.

Studies using aromatic pathway mutants suggest that loss of the capacity for aromatic biosynthesis destroys the virulence of pathogenic bacteria. Aromatic pathway mutants of *Salmonella typhimurium* and *Salmonella typhi* have been tested as attenuated strains that have potential as live vaccines and vaccine carriers (Fairweather et al., 1990; Hone et al., 1991). For example, a *S. typhimurium aroA* mutant has been used as a live carrier to immunize mice against tetanus. The mutant bearing a plasmid that expresses a fragment of the tetanus toxin when used as a live vaccine administered intravenously was able to induce immunity in mice against a lethal tetanus toxin challenge (Fairweather et al., 1990). *Salmonella typhi aroC aroD* double-mutants were shown to be attenuated in mice (Hone et al., 1991), found to be immunogenic in human volunteers, and may be used as vaccines for typhoid fever (Hone and Levine, 1990).

Neisseria gonorrhoeae is an obligate human pathogen, and is the causative organism of gonorrhea. The human host defines its natural environment. Two phenomena, occurring among natural (clinical) isolates of *N. gonorrhoeae*, and affecting

aromatic biosynthesis have attracted our attention. In one case, three clinical isolates were recovered that were auxotrophic for the three aromatic amino acids. The three strains isolated at the Victoria Hospital Corporation (Canada), VHC 3102, 3103 and 3104, were isolated following Aztreonam treatment failure of a single male/female contact pair of patients (A.T. Hendry, personal communication).

In another report (Hendry and Dillon, 1984), up to 1% of over 1000 isolates were found to be sensitive to growth inhibition by phenylalanine. Each of these phenomena have been the subject of preliminary studies in our laboratory.

The above auxotrophic isolates are examples of an unusually broad phenomenon seen among natural isolates of *N. gonorrhoeae*. Over 90% of isolates have been found to be auxotrophic for one or more of a combination of certain amino acids, nucleotide bases, and vitamins (Catlin, 1973; Carifo and Catlin, 1973; Hendry and Stewart, 1979). Besides a general requirement for cystine or cysteine among all isolates, strains could be distinguished with specific requirements for proline, arginine, methionine, histidine, lysine, hypoxanthine, uracil, and thiamine. Indeed, the prevalence of this nutritional heterogeneity allowed for a method of typing *N. gonorrhoeae* isolates into groups, or auxotypes. Auxotyping has been widely used for epidemiological tracking.

The existence of three aromatic auxotrophs (Aro$^-$) appeared paradoxical to the argument that aromatic biosynthesis is important to the survival of the bacterium. However, several aspects are worthy of consideration in this regard. First, the Aro$^-$ strains are not tight auxotrophs (A. T. Hendry, personal communication). They show only a partial requirement for tryptophan and no requirement for the vitamin-like compounds 4-aminobenzoic acid and 4-hydroxybenzoic acid. Secondly, among the several hundreds of auxotrophic isolates these are the only Aro$^-$ strains. Moreover, since they were isolated from the same patient it is very likely that they are sibling strains. Thus, the occurrence of these auxotrophs is probably unique, and reflects some exceptional circumstance that allowed for their selection.

As a first step towards investigating these phenomena our laboratory initiated study of aromatic biosynthesis in *N. gonorrhoeae*. A general outline of aromatic biosynthesis in this bacterium was presented (Berry et al., 1987). In this dissertation, data from more detailed examinations of some of the early pathway enzymes are presented which considerably expand information on the enzymology of the pathway in *N.gonorrhoeae*.

The Shikimate Pathway for Biosynthesis of Aromatic Amino Acids

The biosynthesis of phenylalanine, tyrosine and tryptophan, occurs (Fig. 1-1) from a sequence of reactions

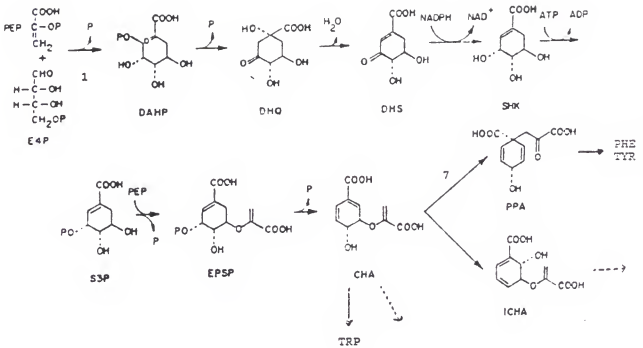


Fig. 1-1. The "main" trunk of the shikimic acid pathway. PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid; SHK, shikimic acid; S3P, shikimic acid 3-phosphate; EPSP, 5-enolpyruvoylshikimic acid 3-phosphate; CHA, chorismic acid; ICHA, isochorismic acid; PPA, prephenic acid; PHE, phenylalanine; TYR, tyrosine; TRP; tryptophan; P, inorganic phosphate. Dashed arrows indicate further branching. In this regard ICHA is now being recognized as an important precusor to a variety of aromatic compounds. DAHP is drawn as the open chain ketone for convenience in visualizing relationships between carbon atoms, although it reacts as a pyranose structure. 1 is DAHP synthase, and 7 is chorismate mutase. The pathways to PHE, TYR and TRP involve a sequence of reactions.

that begins with products of carbohydrate metabolism, erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) and flows *via* shikimic acid to the amino acids and several other pre-aromatic and aromatic compounds [see Bentley, 1990, for a comprehensive review]. The precursors E4P and PEP are condensed in the first step to generate the only acyclic molecule, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), in the entire sequence of reactions that succeed this step. Chorismic acid is the last common precursor in the synthesis of the phenylalanine, tyrosine, tryptophan, 4-hydroxybenzoic acid, and 4-aminobenzoic acid. Chorismic acid offers a highly flexible skeleton that allows several molecules (e.g. ubiquinones, antibiotics, and folic acid) to be derived from it by facile rearrangements, substitutions and eliminations. Chorismic acid is also isomerized to isochorismic acid (Fig. 1-1), from which are produced precursors to benzenoid quinones, and siderophore precursors such as salicylic acid and 2,3-dihydroxybenzoic acid (Bentley, 1990). The pathway as far as chorismic acid is common to the synthesis of the three aromatic amino acids and these other aromatic compounds. Mutants blocked in the common pathway show requirements not only for the aromatic amino acids but also for 4-aminobenzoic acid and 4-hydroxybenzoic acid. Since the quantitative requirement of the latter compounds is very low, tight mutations are essential to cause auxotrophy for these metabolites.

The post-chorismate pathway is quite diverse in nature, and alternative routings to phenylalanine and tyrosine exist (Fig. 1-2). It has been shown by extensive study that various permutations and combinations of the enzymes shown in Fig. 1-2 exist in nature, as depicted in Fig. 1-3 (Byng et al., 1982).

The shikimate pathway has a large carbon-flow and is energetically expensive. Regulation of carbon-flow in the common trunk of the pathway is of great importance and exhibits different patterns that can be related to the structure of the post-chorismate pathway (Jensen and Hall, 1982). The first enzyme, DAHP synthase, is always the focal point of regulation since its reaction is irreversible and commits carbon into the pathway.

The Aromatic Biosynthetic Pathway in *N. gonorrhoeae*.

A general outline of aromatic biosynthesis in *N. gonorrhoeae* was provided by the study of Berry et al. (1987). A single species of the first enzyme, DAHP synthase, which was inhibited by phenylalanine (DAHP synthase-Phe; DS-Phe) was identified. The post-chorismate pathway was found to be relatively simple in its construction. *N. gonorrhoeae* preferentially utilized the 4-hydroxyphenylpyruvate and phenylpyruvate routes to tyrosine and phenylalanine, respectively. The ubiquitous bi-functional P-protein, consisting of the activities of chorismate mutase and prephenate dehydratase, was found. The activity of the

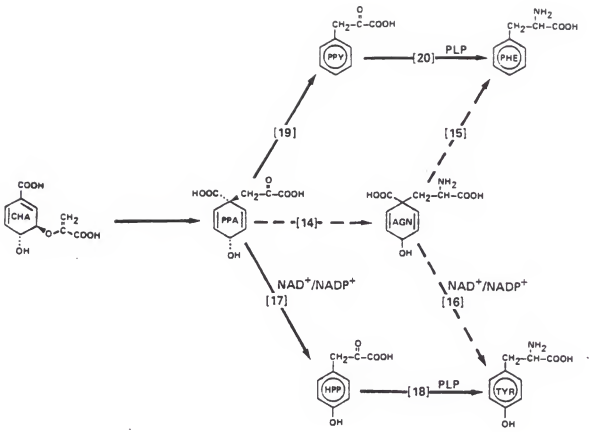


Fig. 1-2. The alternate fates of prephenate *en route* phenylalanine and tyrosine. PPA, prephenate; AGN, arogenate; PPY, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate; PHE, phenylalanine; TYR, tyrosine; PLP, pyridoxal phosphate. CHA is converted to PPA by chorismate mutase. **14** prephenate aminotransferase; **19** prephenate dehydratase; **20** phenypyruvate aminotransferase; **17** prephenate dehydrogenase; **18** 4-hydroxyphenylpyruvate aminotransferase; **15** arogenate dehydratase; **16** arogenate dehydrogenase. Cyclohexadienyl dehydratase (CDT) is capable of performing steps catalyzed by **15** and **19**, while cyclohexadienyl dehydrogenase (CDH) is capable of performing steps catalyzed by **16** and **17**.

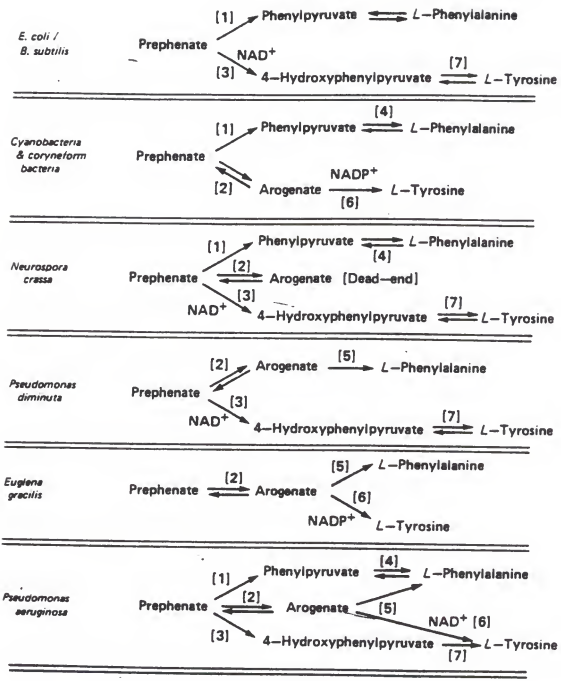


Fig. 1-3. Diversity of the pathways leading to *L*-phenylalanine and *L*-tyrosine. Enzymes along these routes are numbered as follows: 1 prephenate dehydratase, 2 prephenate aminotransferase, 3 prephenate dehydrogenase, 4 phenylpyruvate aminotransferase, 5 arogenate dehydratase, 6 arogenate dehydrogenase, 7 4-hydroxyphenylpyruvate aminotransferase. [Reproduced from Byng *et al.* (27)].

prephenate dehydratase of the P-protein was strongly (~21-fold) stimulated by tyrosine. A cyclohexadienyl dehydrogenase that had a strong preference for prephenate and an absolute specificity for NAD^+ was demonstrated. This activity along the tyrosine route was not allosterically regulated.

Tyrosine directly controls phenylalanine biosynthesis since the prephenate dehydratase is dependent on tyrosine for activation. Phenylalanine in turn governs overall aromatic biosynthesis by exerting allosteric control over DAHP synthase. The DAHP synthase appeared to be the focal point of regulation within the pathway, and the only control point for tyrosine biosynthesis. Consequently, mutants selected for resistance to growth inhibition by phenylalanine and tyrosine analogs are either insensitive to feedback inhibition or derepressed at the level of DAHP synthase (R. K. Bhatnagar, unpublished observations). Thus, DAHP synthase was chosen as the enzyme for further study at the outset of this project.

Key Initial Observations Fundamental to the Formulation of Objectives

DAHP synthase has been the focal point of interest for two reasons: (i) because of its possible role in the sensitivity of some clinical isolates to growth inhibition by phenylalanine (Bhatnagar et. al, 1989), and (ii) crude extracts from Aro^- strains lacked apparent activity for DAHP synthase (R. K. Bhatnagar, unpublished observation).

Furthermore, I obtained new results following hydroxylapatite chromatography of crude extract preparations from strains of *N. gonorrhoeae*. In contrast to the single peak of DAHP synthase reported by Berry et. al (1987), three peaks of activity for the enzyme were resolved. Two major species, designated DS-Phe^{L} (for leading peak) and DS-Phe^{T} (for trailing peak), exhibited feedback-inhibition by phenylalanine. The third and minor species was insensitive to inhibition by any metabolite known to inhibit other DAHP synthases.

Chromatography of crude extracts from the Aro^{-} strains showed them to possess DS-Phe^{T} alone. This was in contrast to the apparent lack of activity for DAHP synthase in crude extracts. The DS-Phe^{T} activity seemed inconsistent with the auxotrophy of the Aro^{-} strains. The possibility that DAHP produced by DS-Phe^{T} might be specifically diverted, by some enzyme, away from aromatic amino acid biosynthesis to an unknown pathway was considered. This would imply that DS-Phe^{L} was the "biosynthetic" species. However, only dehydroquinate synthase, the enzyme that follows DAHP synthase in the aromatic amino acid biosynthetic pathway, was found.

A second possibility to account for the lack of DS-Phe^{L} in Aro^{-} was that a common-pathway enzyme other than DAHP synthase was missing in the Aro^{-} strains and that the lack of DS-Phe^{L} was an indirect effect reflecting the loss of a required (obligatory) protein-protein interaction. Indeed,

each of the three Aro$^-$ strains was found to lack activity for shikimate dehydrogenase. The pleiotropic loss of DS-Phe^L and shikimate dehydrogenase was presumptive evidence that DS-Phe^L and shikimate dehydrogenase formed a dissociable complex, or existed as a bifunctional protein.

The minor species of DAHP synthase was first resolved by chromatography in the Aro$^-$ strains, since in the prototrophic strains this activity overlapped with DS-Phe^L making it less obvious. One possibility was that this minor species was a previously undetected DS-0, an unregulated class of DAHP synthase, thus far characterized only in *Acinetobacter calcaoceticus* and *Oceanospirrulum minutulum* (Ahmed et al., 1986). Another possibility was that it might not be a DAHP synthase at all, but rather a broad-specificity type of 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase, previously reported only in higher plants (Doong et al., 1991).

Project Objectives

The objectives of this study were, thus, to answer the following questions.

1). Are the activities of DS-Phe^L and shikimate dehydrogenase (SDH) associated? If so, does the DS-Phe^L-SDH association represent (i) a bifunctional protein or (ii) a dissociable complex. Option (i) would imply that two DS-Phe genes were present, while option (ii) could be explained by a single gene product, since the two DS-Phe forms could

represent associated and dissociated forms of this gene product. These experiments are discussed in Chapter 2.

2). What is the nature of the third minor DS "isozyme"? Is it a DS-0 or KDOP synthase? If it is a KDOP synthase, does it make DAHP? This question was answered by studying the nature of the product formed from the reaction of this enzyme with E4P. These experiments are discussed in Chapter 3.

CHAPTER 2
PROTEIN-PROTEIN INTERACTIONS AMONG THE ENZYMES IN THE COMMON PATHWAY OF AROMATIC BIOSYNTHESIS IN *NEISSERIA GONORRHOEAE*.

Introduction

3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase (recommended name, EC 4.1.2.15, phospho-2-dehydro-3-deoxyheptonate aldolase; trivial name, DAHP synthase) catalyses the condensation of E4P and PEP to produce 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP). The enzyme, which sits at the juncture of carbohydrate metabolism and aromatic biosynthesis, commits carbon irreversibly towards aromatic biosynthesis. The activity of the enzyme is almost always regulated (Byng et al., 1982). This protein is now known to exemplify the largest number of allosteric regulatory patterns thus far described for any one protein (Bentley, 1990). These include: (1) isoenzymic, (2) sequential, (3) concerted (synergistic or co-operative), (4) cumulative, and (5) unimetabolite patterns of allosteric inhibition (27). *Escherichia* and *Salmonella* genera have isoenzymic forms of DAHP synthase, each individually inhibited by phenylalanine (DS-Phe), tyrosine (DS-Tyr), and tryptophan (DS-Trp). They are encoded by the genes, *aroG*, *aroF*, *aroH*, respectively (Pittard, 1987). Repression control at the transcriptional

level, best studied in *E. coli*, adds another dimension to the control of enzymic activity (Pittard, 1987).

An unregulated species of DAHP synthase, DS-0, has been characterized in two bacterial genera, *Acinetobacter calcaoceticus* and *Oceanospirillum minutulum* (Ahmad et al., 1986).

In two bacteria, *Bacillus subtilis* and *Brevibacterium flavum*, DAHP synthase has been shown to exist with chorismate mutase as a bifunctional protein that in turn is part of a multifunctional complex. In *B. subtilis*, a DS-PPA(CHA) exists as a bifunctional polypeptide with the activity of chorismate mutase (Lorence and Nester, 1967). Both prephenate and chorismate inhibit the activity of the DAHP synthase with prephenate being the more potent effector. In effect, the active site of the chorismate mutase acts as the allosteric site for the DS-PPA(CHA) (Llewellyn et al., 1980). This protein forms a trifunctional complex with shikimate kinase (Nakatsukasa and Nester, 1972). In *Brevibacterium flavum*, a DS-Phe/Tyr species is part of a bifunctional protein with one of two subunits of chorismate mutase (CM-A) (Shiio and Sugimoto, 1979). Phenylalanine and tyrosine inhibit the activity of the DAHP synthase synergistically. This bifunctional protein in turn exists as a component of a trifunctional complex with the other subunit of chorismate mutase (CM-B).

In *N. gonorrhoeae*, a single DAHP synthase exists which is feedback inhibited by phenylalanine (Berry et al., 1987). In this chapter, data are presented that suggest that the DS-Phe participates in a multifunctional complex with the common pathway enzymes shikimate dehydrogenase and dehydroquinate synthase. All three activities are dissociable. This provides another example of a multienzyme complex involving DAHP synthase. It is not known at this time whether other enzymes are associated with this complex.

Materials and Methods

Chemicals and Biochemicals.

Erythrose-4-phosphate was prepared according to 'Procedure B' of Ballou (1963) except that the monosodium salt of glucose-6-phosphate was used, and the sulfuric acid treatment of the salt was omitted. The concentration of erythrose-4-phosphate was estimated by using a partially purified preparation of DAHP synthase from *N. gonorrhoeae* ATCC 27630. Dehydroquinate was synthesized according to the procedure of Haslam et al. (1963). The product was obtained as an oil that could be dried to an extremely hygroscopic solid, but could not be crystallized. The free acid was dissolved in water (to a concentration of 175 mM), the pH adjusted to 7.0, and the solution stored at -80°C. It was found to contain ~8% of dehydroshikimic acid by its absorbance at 234 nm.

DAHP was isolated from the culture supernatant of an overproducing mutant of *E. coli* according to the procedure of Mehdi et al. (1987). The mutant strain *E. coli* JB5 was a gift from Jeremy Knowles (Harvard University).

The trisodium salt of phosphoenolpyruvate (PEP) was purchased from Sigma Chemical Co. (St. Louis, Mo). Dithiothreitol (DTT) was purchased from Research Organics Inc. (Cleveland, Ohio). Hydroxylapatite (Bio-gel HTP) was purchased form Bio-Rad (Rockville Centre, NY). Bio-Gel A-0.5 m, 100-200 mesh, (exclusion limit 500,000 daltons) was purchased from Bio-Rad as a fully hydrated suspension in 10 m*M* Tris-EDTA buffer. This buffer was replaced with appropriate buffers after packing the column.

Bacterial Strains, Media and Growth Conditions.

The bacterial strains used in this chapter are presented in Table 2-1. These strains were grown in the minimal medium formulated by Hendry (1983). Growth was followed at 525 nm by measuring the optical density of a 5-ml sample of the culture at regular intervals to late-exponential phase. Supplementation of media to appropriate concentrations with required components was done through a sterilizing membrane, after the complete medium was made. All strains were provided by Dr. A. T. Hendry, Hamilton General Hospital, Ontario, Canada.

Table 2-1. List of *N. gonorrhoeae* strains used.

Strain[a]	Phenotype[b]
2013 (ATCC 27630)	Pro^-, Phe^R, taken as "wild type"
2013-7	mFP^R, spontaneous mutant from strain 2013
2013-8	pFP^R, spontaneous mutant from strain 2013
2011 (ATCC 27628)	Prototrophic, Phe^S
HGH 154	Prototrophic, Phe^S
VHC 3102	Aro^-, Ura^-, Cit^-
VHC 3103	Aro^-, Ura^-, Cit^-

[a]All strains were provided by Dr. A. T. Hendry, Hamilton General Hospital (HGH), Ontario, Canada. VHC 3102/3103 were originally isolated in Victoria Hospital Corporation, Ontario, Canada.

[b]Phe^R, resistant to growth inhibition by phenylalanine; Phe^S, sensitive to growth inhibition by phenylalanine; Aro, Phenylalanine + Tyrosine + Tryptophan; Ura, Uracil; Cit, Citrulline; mFP, m-fluorophenylalanine; pFP, p-fluorophenylalanine.

Cells in late-exponential phase of growth were harvested by centrifugation at 4°C, washed once with Buffer A (20 m*M* K-phosphate, pH 7.0, 1.0 m*M* DTT) and stored at -80°C until used.

Preparation of Crude Extracts.

Cells were resuspended by thawing the frozen cells in Buffer B (100 m*M* K-phosphate, pH 7.0, 1.0 m*M* DTT) at room temperature. A buffer-to-cell ratio (v:w) of 2:1 was used. Cells were broken in a French pressure-cell at 16,000 psi. The suspension was centrifuged at 150,000 x g for 35 min and the supernatant collected as the crude extract. Longer centrifugation times caused a progressive sedimentation of DAHP synthase activity. Extracts were desalted by passage through an Econo Pac 10DG (Bio-Rad) desalting column. All operations were conducted at 4°C.

Chromatography.

Hydroxylapatite chromatography.

Typically ~75 mg (total protein) of the desalted crude extract in Buffer A was loaded on a 1.5 x 20 cm column of hydroxylapatite equilibrated in Buffer A. After the unbound protein was washed with two bed-volumes of Buffer A, adsorbed protein was eluted with a linear gradient between Buffer A and 300 m*M* K-phosphate, pH 7.0, containing 1.0 m*M* DTT, in a total volume of 340 ml. Fractions of 2.2 ml were collected.

DEAE-cellulose chromatography.

A 75- to 100-mg portion of the desalted crude extract in Buffer B was loaded onto a 1.5 x 20 cm column of DEAE-cellulose (DE-52, Fisher Scientific, Orlando, FL). The column was then eluted with 2 x bed-volume of Buffer B and fractions of 2.2 ml were collected.

Gel-filtration chromatography.

Bio-Gel A-0.5m. Gel-filtration was performed on a 2.8 x 96 cm column of Bio-Gel A-0.5m, 100-200 mesh. The column was equilibrated with Buffer B. A 5-ml sample in Buffer B was loaded and the proteins were eluted with Buffer B at a flow-rate of 1 ml per min into fractions of 4 ml. PEP was included, where indicated, at 0.75 m*M* in Buffer B. The column was calibrated using the following standards from the MW-GF-200 Molecular weight kit from Sigma Chemical Co.: cytochrome c, 12,400 daltons; carbonic anhydrase, 29,000 daltons; albumin (bovine serum), 66,000 daltons; alcohol dehydrogenase, 150,000 daltons; β-amylase (sweet potato), 200,000 daltons. The void volume was determined using Blue dextran.

Sephadex G-100. *Column A* : A 3 x 39 cm column of Sephadex G-100 Superfine (10-40 μm particle size; Sigma Chemical Co.) was equilibrated in Buffer B. A 3.0-ml sample was loaded and the proteins were eluted with Buffer B, at a flow-rate of 10 ml per hour, into fractions of 3 ml. PEP was included in Buffer B, when indicated, at 0.75 m*M*.

Column B: A 3 x 55 cm column of Sephadex G-100 (40-120 μm particle size; Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated with Buffer B. A 3.5-ml sample was loaded and proteins were eluted in Buffer B at a flow-rate of 30 ml per hour, into fractions of 3 ml. PEP was included in Buffer B, when indicated, at 0.75 m*M*.

Both column A and column B were calibrated using the following proteins as standards: cytochrome c, 12,400 daltons; albumin (chicken egg), 43,000 daltons; and albumin (bovine serum), 66,000 daltons.

Enzyme Assays.

DAHP synthase was assayed as described by Jensen and Nester (1966). Standard reaction mixtures contained 1.5 m*M* PEP, 1.0 m*M* of E4P and 0.5 m*M* $MnSO_4$, and appropriate aliquots of enzyme, incubated at 37°C for 20 min. Activity is expressed as A_{549} where a value of 1.0 corresponds to 0.3 m*M* DAHP formed. An ϵ value of 5.0 X 10^4 M^{-1} cm^{-1} was used (Mehdi et al., 1987).

Shikimate dehydrogenase was measured in a continuous assay by following the appearance of NADPH fluorometrically - Ex: 360 nm, Em:420 nm (Hall et al., 1982). Standard reaction mixtures contained 0.75 m*M* shikimic acid, 0.5 m*M* $NADP^+$, and appropriate aliquots of enzyme in a 400-μl reaction in Buffer B. Reactions were initiated by addition of shikimate to the mixture incubated at 37°C in a thermo-cuvette.

Dehydroquinate synthase was assayed by following the disappearance of substrate DAHP with the thiobarbituric acid (TBA) assay method for DAHP synthase. Reaction mixtures contained 0.3 m*M* DAHP, 50 μM NAD^+ and 0.5 m*M* Co^{+2}, and were incubated at 37°C for 20 min.

A coupled assay, which obviated the need to isolate DAHP, was also used. Dehydroquinate synthase was assayed by coupling to the DAHP synthase reaction using the property that dehydroquinate synthase from *N. gonorrhoeae*, when present in a DAHP synthase reaction mixture, can use DAHP as it is formed. A DAHP synthase reaction mixture was prepared containing 0.8 m*M* PEP, 1.0 m*M* E4P and 0.5 m*M* $MnSO_4$, and an aliquot of partially purified DAHP synthase from chromatography on hydroxylapatite that would give an A_{549} of 1.0-1.5. An aliquot of the fraction to be assayed was added and the reaction incubated at 37°C for 20 min. A control reaction was prepared identically except that the dehydroquinate synthase was omitted. The loss of DAHP measured against the control was taken as a measure of dehydroquinate synthase. For low levels of activity, crude DAHP was first accumulated by proportionately scaling up the DAHP synthase reaction. After ~1.0 hr of incubation at 37°C, the DAHP synthase was destroyed by heating to 60°C for 5 min and the resulting solution used as a source of substrate, DAHP, for dehydroquinate synthase. Activity was measured by following the disappearance of DAHP, in the presence of 50 μM

NAD^+ and 0.5 m*M* Co^{+2}. In all cases the formation of the final product, dehydroquinate, was verified using a highly purified preparation of a bi-functional dehydroquinate dehydratase:shikimate dehydrogenase from *Nicotiana silvestris* that was a gift from Carol Bonner.

Dehydroquinate dehydratase was assayed by following the formation of dehydroshikimic acid spectrophotometrically at 234 nm, at 37°C (Coggins et al., 1987). An ϵ value of 12,000 M^{-1} cm^{-1} was used for dehydroshikimic acid. The reactions contained 0.25 m*M* of dehydroquinic acid and an appropriate amount of enzyme in a final volume of 400 μl.

Results and Discussion

Fig. 2-1 shows the activity profile for the DAHP synthase eluted from a hydroxylapatite column loaded with crude extract from *N. gonorrhoeae* strain HGH 154. This is the same strain used by Berry et al. (1987) in the initial description of the pathway. Two distinct peaks of activity were eluted off the column. Essentially identical results are obtained with the strain ATCC 27630 (2013) and strain ATCC 27628 (2011).

The activities of both forms of DAHP synthase were inhibited by phenylalanine. At a fixed concentration of the substrates, 1.0m*M* erythrose-4-phosphate (E4P) and 1.0 m*M* phosphoenolpyruvate (PEP), the two forms showed very similar responses to varying concentrations of phenylalanine (Fig. 2-2A). Fig. 2-2B shows that identical results were obtained

Fig. 2-1. Elution profiles of DAHP synthase and shikimate dehydrogenase from hydroxylapatite chromatography of *N. gonorrhoeae* strain HGH 154. Columns were run as described in the section on Materials and Methods. Activity for DAHP synthase is reported as A_{549}. DS^L and DS^T refer to the leading and trailing peaks, respectively, of DAHP synthase activity. The distribution of eluted protein was monitored as A_{280}. The concentration of the phosphate gradient at the peak fractions is indicated.

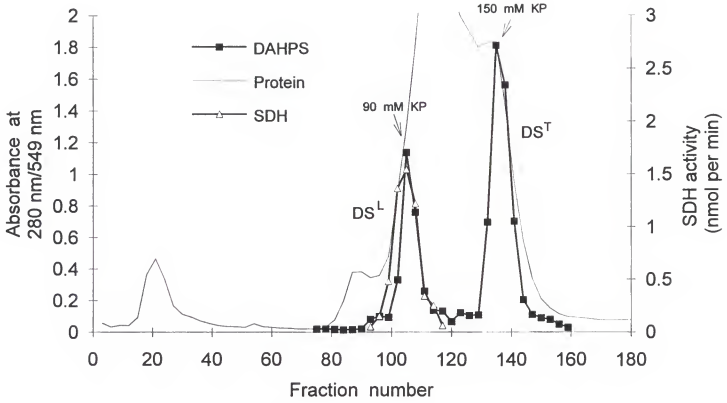
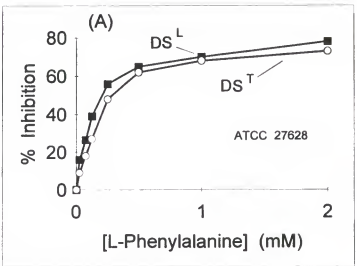
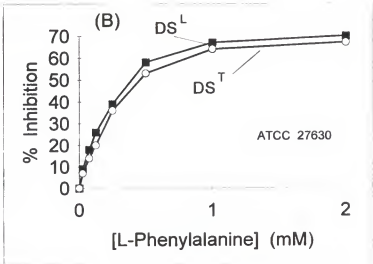
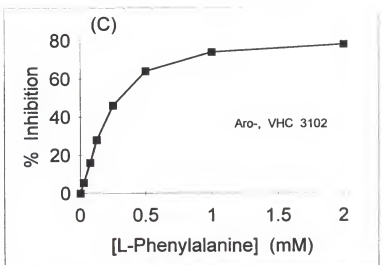

DAHPS
Protein
SDH
150 mM KP
90 mM KP
DS^T
DS^L
Absorbance at 280 nm/549 nm
SDH activity (nmol per min)
Fraction number
0
20
40
60
80
100
120
140
160
180
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
0.5
1
1.5
2
2.5
3

Fig. 2-2. Inhibition of DAHP synthase by *L*-phenylalanine. Reactions contained 1.0 mM each of E4P, PEP, and 0.5 m*M* Mn^{+2}, and the indicated amounts of *L*-phenylalanine. DAHP synthase fractions from hydroxylapatite chromatography of **(A)** strain ATCC 27628, and **(B)** strain ATCC 27630, separated under conditions similar to those depicted in Fig. 2-1, and **(C)** strain VHC 3102 as depicted in Fig. 2-3 were used. DS^{-L} and DS^{T} refer to the leading and trailing peaks, respectively, in Fig. 2-1.


(A)
DS L
DS T
ATCC 27628
% Inhibition
80
60
40
20
0
0
1
2
[L-Phenylalanine] (mM)


(B)
DS L
DS T
ATCC 27630
% Inhibition
70
60
50
40
30
20
10
0
0
1
2
[L-Phenylalanine] (mM)


(C)
Aro-, VHC 3102
% Inhibition
80
60
40
20
0
0
0.5
1
1.5
2
[L-Phenylalanine] (mM)

with strain ATCC 27630 (2013). No other metabolite tested (tyrosine, tryptophan, prephenate, phenylpyruvate or chorismate) was found to exert an effect on the activities of the two forms. The other enzyme activity depicted in the profile, shikimate dehydrogenase, is discussed later.

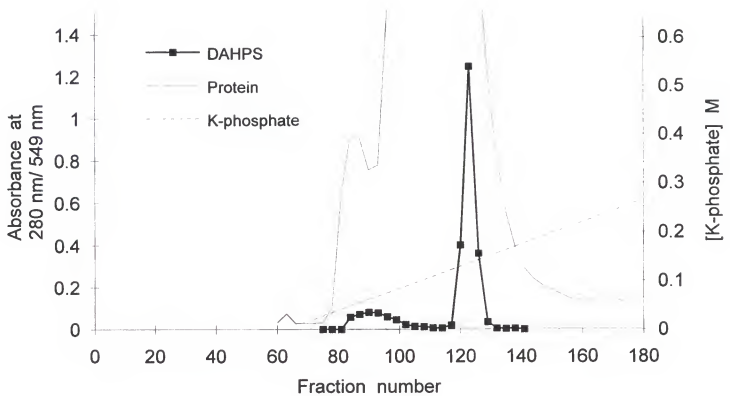
Fig. 2-3 shows the activity profile for DAHP synthase after chromatography of crude extracts of strain VHC 3102 (Aro^-) on hydroxylapatite under the same conditions. A single major peak of activity for DAHP synthase eluted off the column that was approximately coincident (140 m*M* K-phosphate) in its position in the gradient with the position of the trailing peak of activity shown in Fig. 2-1. The small peak activity seen in the profile of Fig. 2-1 proved to be a KDOP synthase and is discussed in the next chapter.

As expected, the activity of the DAHP synthase was inhibited by phenylalanine. Varying concentrations of phenylalanine produced a response curve (Fig. 2-2C) very similar to that shown in Figs. 2-2A and 2-2B.

The basis for the Aro^- phenotype became apparent when VHC 3102 was shown to lack shikimate dehydrogenase activity. No activity was detected in crude extracts or following hydroxylapatite chromatography. Strain VHC 3103, another of the three Aro^- isolates was identical to VHC 3102 in all of the above mentioned characteristics.

Since strain VHC 3102 exhibits an Aro^- phenotype and requires the presence of the aromatic amino acids in the

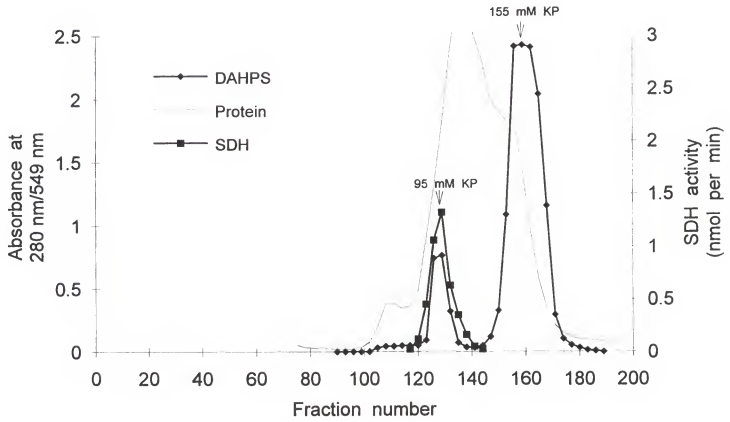
Fig. 2-3. Elution profile for DAHP synthase from hydroxylapatite chromatography of crude extracts of VHC 3102. Extracts were chromatographed as described in the section on 'Materials and Methods'. DAHP synthase activity is reported as A_{549}. The dashed diagonal line shows the progression of the K-phosphate gradient. The elution of protein was monitored as A_{280}.


DAHPS
Protein
K-phosphate
Absorbance at 280 nm/ 549 nm
[K-phosphate] M
Fraction number
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0.1
0.3
0.5
20
40
60
80
100
120
140
160
180

growth medium the possibility was considered that the leading peak might be selectively repressed in this strain, hence, yielding only one peak of activity in the profile. Strain 2013 was grown in the presence of phenylalanine, tyrosine and tryptophan (at 100 μg ml^{-1} each) in the growth medium. Crude extracts were chromatographed under conditions identical to those used for strain VHC 3102 and the results are shown in Fig. 2-4. The amino acids had no effect on the leading peak of DAHP synthase, or on the activity of shikimate dehydrogenase, when compared with the same strain grown on medium without the amino acids.

Thus, it seemed a mechanism linked the loss of shikimate dehydrogenase with the absence of the leading peak of DS-Phe in strain VHC 3102. The chromatographic profiles of the two enzymes in strain HGH 154 (Fig. 2-1) and strain 2013 (Fig. 2-4) revealed the shikimate dehydrogenase activity to be co-incident with the leading peak of DAHP synthase. This raised the possibility that the two enzymes may be associated either as a bifunctional polypeptide or as a bi-functional complex. While the first option would imply two genes for DAHP synthase, the second option could be explained by a single gene product, since the two activities of DAHP synthase could represent associated and dissociated forms of DAHP synthase. Indeed, in the rest of this chapter data are presented that are consistent with the presence of a complex consisting of

Fig. 2-4. Hydroxylapatite chromatography of crude extracts of strain 2013 grown in the presence of phenylalanine, tyrosine and tryptophan. Amino acids were present at 100 μg ml^{-1} in the growth medium. Extracts were prepared and chromatographed as described in the section on 'Materials and Methods'. The concentration of the phosphate gradient at the peak fractions is indicated. Elution of protein was monitored as A_{280}. DAHP synthase (DAHPS) activity is expressed as A_{549}.


155 mM KP
95 mM KP
DAHPS
Protein
SDH
Absorbance at 280 nm/549 nm
SDH activity (nmol per min)
Fraction number
2.5
2
1.5
1
0.5
0
3
2.5
2
1.5
1
0.5
0
0 20 40 60 80 100 120 140 160 180 200

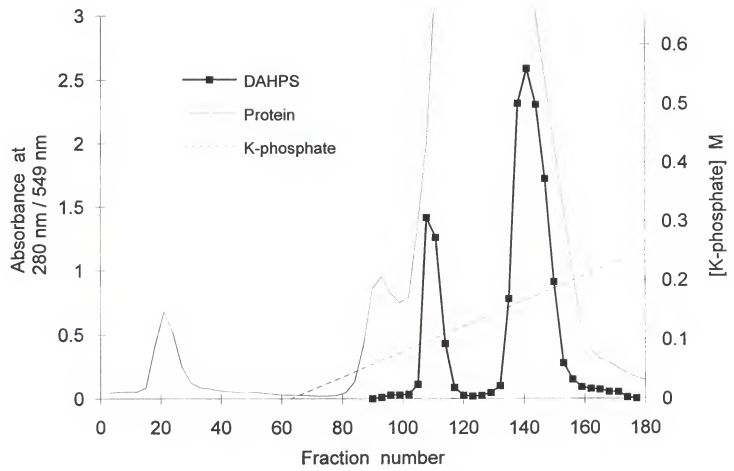
the activities of DAHP synthase and shikimate dehydrogenase. The two activities are dissociable and the two enzymes are active as monomers.

Are the Two DAHP Synthases the Product of the Same Gene?.

Data from the analysis of certain mutants suggest that the two species of DAHP synthase arise from the same gene. Several spontaneous mutants have been selected for resistance to the metabolite analogs m-fluorophenylalanine (mFP) and m-fluorotyrosine. Crude extracts of a mFP mutant (2013-7) were chromatographed on hydroxylapatite as in Fig. 2-1 to give the results shown if Fig. 2-5. Two peaks of activity for DAHP synthase are seen. Both the forms of the enzyme were, however, simultaneously desensitized to the inhibitory effect of phenylalanine. The concurrent loss of inhibition by phenylalanine suggests both forms originate from the same gene. These data are consistent with earlier unpublished observations (R. K. Bhatanagar) using crude extracts of a other mutants where DAHP synthase activity was found to be totally insensitive to phenylalanine.

This conclusion is also consistent with the fact that in the parent strain 2013 both the enzyme forms are similar in their response to the effects of varying concentrations of phenylalanine.

Fig. 2-5. Hydroxylapatite chromatography of crude extracts of strain 2013-7, a spontaneous mFP-resistant mutant selected from strain 2013. The column was run as described in the section on 'Materials and Methods'. The diagonal dashed line shows progression of the gradient. DAHP synthase activity is reported as A_{549}. Elution of protein is monitored as A_{280}.


DAHPS
Protein
K-phosphate
Absorbance at 280 nm / 549 nm
[K-phosphate] M
Fraction number
0
0.5
1
1.5
2
2.5
3
0.1
0.2
0.3
0.4
0.5
0.6
0
20
40
60
80
100
120
140
160
180

The Monomeric Form of DAHP Synthase.

Chromatography of crude extracts of strain 2013 over a DEAE-cellulose column equilibrated in Buffer B (see section on Materials and Methods) resulted in the elution of DAHP synthase activity in the wash fractions (Fig. 2-6). All the DAHP synthase activity was associated with these fractions and no detectable activity was found to be bound to the column when eluted by the gradient (0-400 m*M* KCl in Buffer B). These conditions were found to be a quick and convenient method to pool all of the DAHP synthase as one peak of activity while achieving some degree of protein purification. Also, this helped separate shikimate dehydrogenase activity from that of DAHP synthase.

The DAHP synthase activity that was separated from the shikimate dehydrogenase was concentrated by ultrafiltration on a PM-10 membrane and rechromatographed on a calibrated Sephadex G-100 gel-filtration column equilibrated with the Buffer B (Fig. 2-7). The enzyme eluted as a single peak of activity with a relative molecular mass (M_r) of ~35,000 (Fig. 2-7, peak a).

Phosphoenolpyruvate, one of the substrates of the DAHP synthase, was found to affect the native state of the enzyme. The enzyme recovered from the above gel filtration was concentrated by ultrafiltration to 5.5 ml. PEP was added to a final concentration of 0.75 m*M* and the preparation incubated at 4°C for ~ 1 hour. A 3-ml sample was then chromatographed

Fig. 2-6. DEAE-cellulose chromatography of crude extracts of strain 2013. A 72-mg portion of crude extract was loaded on the column and chromatographed as described in the section on 'Materials and Methods' and fractions were assayed for activities of DAHP synthase and shikimate dehydrogenase.

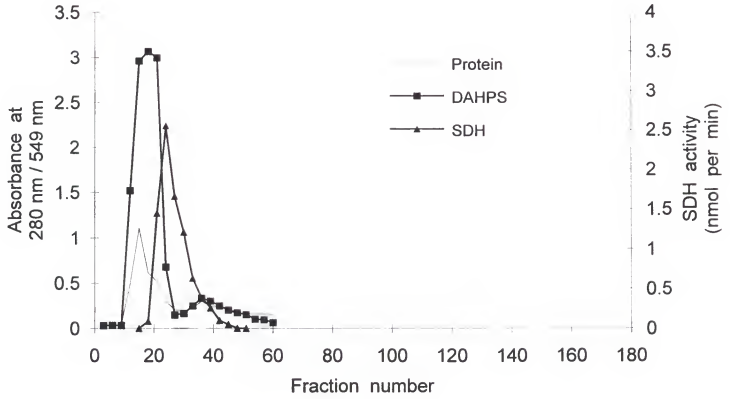

Absorbance at 280 nm / 549 nm
SDH activity (nmol per min)
Fraction number
Protein
DAHPS
SDH
0
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80
100
120
140
160
180

Fig. 2-7. Gel-filtration of DAHP synthase. DAHP synthase activity recovered from the DEAE-cellulose column (Fig. 2-6), as described in the text, was chromatographed on a Sephadex G-100 column as described in the section on 'Materials and Methods' (Column A), to give the elution profile denoted by peak *a*. Peak *b* denotes the effect of PEP on the activity eluted in peak *a*. Activity within peak *a* was pooled, concentrated and rechromatographed on the same column, except that 0.75 m*M* PEP was included in the buffer, to give peak *b*. The two chromatograms have been superimposed for comparison. The V_o of the column was 96 ml. V_e for peak *a* was 150 ml, and for peak *b* was 117 ml. The elution of protein (as A_{280}) is shown for the chromatogram of peak-b alone to emphasize that the shift in peak position is not a general protein effect but "specific" to the activity of DAHP synthase.

Absorbance at 549 nm
(Peak a)
Absorbance at 280 nm
Absorbance at 549 nm
(Peak b)
Elution volume (ml)
12.4 KD
43 KD
66 KD
V_o
a
b
0
0.5
1
1.5
2
2.5
0.1
0.2
0.3
0.4
0.5
0.6
0
50
100
150
200
250
300
350
400
450

on the same Sephadex G-100 column as before, except that PEP was included in the buffer. One peak of activity eluted at an M_r of ~70,000 (Fig. 2-7, peak b). Thus, PEP mediates a monomer-dimer equilibrium of the DAHP synthase. In an earlier study, Bhatnagar et al. (1989) had shown the enzyme to exhibit positive co-operativity. When PEP was the varied substrate sigmoid substrate-saturation curves were obtained and typical non-linear Lineweaver-Burk plots indicative of positive co-operativity were obtained.

Gel Filtration of Crude Extracts Point to the Association of the Activities of DAHP Synthase and Shikimate Dehydrogenase.

With the knowledge of the monomeric M_r of DAHP synthase, the behavior of the enzyme in crude cell-extracts could be followed by gel-filtration. Two conditions were used: chromatography in the presence and absence of 0.75 m*M* PEP in the equilibration buffer. Fig. 2-8 shows the results of gel filtration of crude extracts of strain 2013 on a Bio-gel A-0.5m gel gel-filtration column in the presence of PEP. Two peaks of activity for the DAHP synthase are apparent, one at an M_r of ~150,000 and the other an M_r of ~100,000. Shikimate dehydrogenase was found to co-elute with the latter species of DAHP synthase.

Fig. 2-9 shows the results of an identical experiment that was run in the absence of PEP in the equilibration buffer. Quite surprisingly, two peaks of shikimate dehydrogenase are apparent that are each associated with the

Fig. 2-8. Gel-filtration of a crude extract of strain 2013 on a Bio-gel A-0.5m column. A 50-mg portion in 5 ml was loaded on the column pre-equilibrated with buffer containing 0.75 m*M* PEP, as described in the section on 'Materials and Methods'. V_o represents the void volume. Peak *a* eluted at a V_e of 228 ml (~150 kD) and peak *b* eluted at a V_e of 264 ml (~100 kD). DAHP synthase (DAHPS) and shikimate dehydrogenase (SDH) activities are reported as in Fig. 2-1.

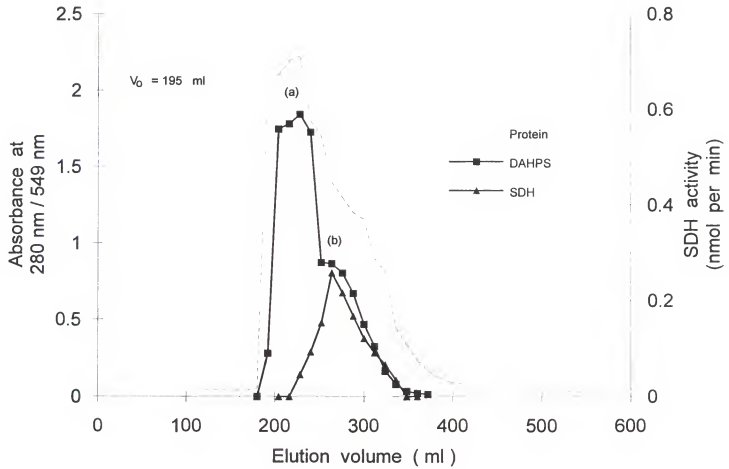
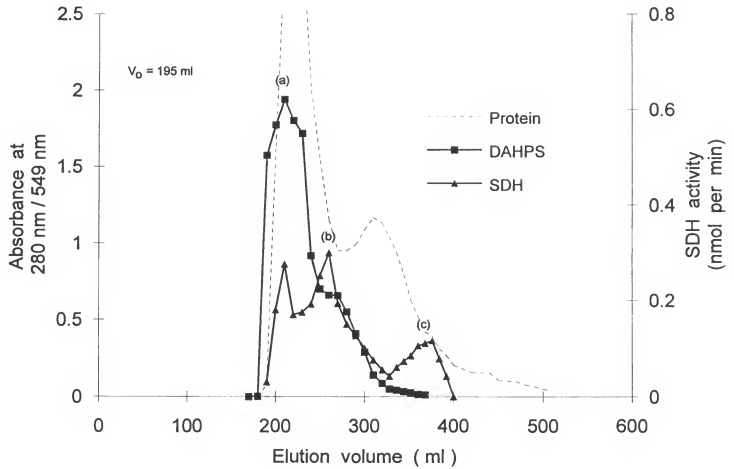

2.5
2
1.5
1
0.5
0
0.8
0.6
0.4
0.2
0
V_o = 195 ml
(a)
(b)
Protein
DAHPS
SDH
Absorbance at 280 nm / 549 nm
SDH activity (nmol per min)
0
100
200
300
400
500
600
Elution volume (ml)

Fig. 2-9. Gel-filtration of a crude extract from strain 2013 on a Bio-gel A-0.5m column. Extracts were chromatographed under the conditions depicted in Fig. 3-7, except that PEP was omitted from the equilibration buffer. The elution volumes were as follows: peak *a*- 210 ml (~185 kD), peak *b*- 264 ml(~100 kD). Peak *c* at ~376 ml (~30 kD) represents the monomeric form of SDH.


2.5
2
1.5
1
0.5
0
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
V_o = 195 ml
(a)
(b)
(c)
Protein
DAHPS
SDH
Absorbance at 280 nm / 549 nm
SDH activity (nmol per min)
Elution volume (ml)

higher and lower molecular weight species. The higher molecular weight species at an M_r of ~175,000 is consistent with the approximate addition of a sub-unit of M_r ~27,000 to the M_r ~150,000 species in Fig. 2-8 (peak *a*). This unit is the monomeric weight of shikimate dehydrogenase (peak *c*, Fig. 2-9) as described in the next section. Thus, the appearance of the new peak of shikimate dehydrogenase at the higher molecular weight position also causes a shift in the position of the DAHP synthase (peak *a*; compared to Fig. 2-8) by an amount equal to the M_r of shikimate dehydrogenase. It, thus, appears that DAHP synthase and shikimate dehydrogenase exist as components of a complex of M_r ~ 180,000. The significance of the differential association of the two activities brought about by PEP is not known.

The Monomeric Nature of Shikimate Dehydrogenase.

The shikimate dehydrogenase that was eluted under the conditions depicted in Fig. 2-6 was further chromatographed to identify its unit molecular weight. Shikimate dehydrogenase binds to a 2'-5'-ADP agarose affinity column and can be released from the gel by elution with its substrate NADP. The enzyme from the DEAE-column (fractions 18-36) depicted in Fig. 2-6, was concentrated by ultrafiltration on a PM-10 membrane and dialyzed against Buffer A and adsorbed on a 1 x 23 cm 2'-5'-ADP agarose column equilibrated with Buffer A. After washing the column with 2 bed-volumes of the equilibration

buffer the shikimate dehydrogenase was eluted with Buffer B containing 0.5 m*M* NADP, into fractions of 2.2ml. The active fractions were pooled and the eluate concentrated by ultrafiltration. The concentrate (3.5 ml) was loaded onto a Sephadex G-100 column (3 x 55 cm) and chromatographed under conditions of 'Column B', as described in the 'Materials and Methods' section. Shikimate dehydrogenase eluted with an M_r of 27,000 (Fig. 2-10).

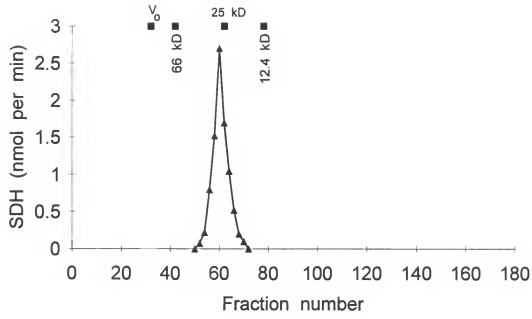
Purification of Shikimate Dehydrogenase.

Cells (40 g) of *N. gonorrhoeae* strain 2013 were resuspended in ~70 ml of Buffer B and broken by single passage through a French pressure-cell at 16,000 psi. The extract was centrifuged at 150,000 x g for 35 min at 4°C and the supernatant collected. A 100 μg each of DNAse and RNAse were added, and the supernatant was dialyzed against Buffer A with three changes over 24 hrs.

DEAE-cellulose chromatography.

The above extract was loaded onto a 2.8 x 49 cm DEAE-cellulose column equilibrated with Buffer A. The unbound protein was washed with two bed-volumes of Buffer A and the column was eluted with a linear gradient between 0-400 m*M* of KCl in Buffer A in a total volume of 1800 ml, while collecting fractions of 8.5 ml. The gradient fractions (#152-163) showing activity for shikimate dehydrogenase were pooled (~95

Fig. 2-10. The monomeric form of shikimate dehydrogenase (SDH). An affinity purified preparation, as described in the text, of SDH from strain 2013 was chromatographed on a Sephadex G-100 column as described in the section on 'Materials and Methods' for 'Column B'. The elution positions of molecular-weight standards are shown; V_o is the void volume.


V_o
25 kD
66 kD
12.4 kD
SDH (nmol per min)
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
140
160
180
Fraction number

ml). Shikimate and fresh DTT were added to a final concentration of 0.5 m*M*, each. The solution was concentrated by ultrafiltration through a PM-10 membrane and the concentrate dialyzed against Buffer A until the conductance of a suitably diluted sample was the same as for Buffer A.

Hydroxylapatite chromatography.

The concentrated preparation from DEAE-cellulose was loaded onto a 2.8 x 17 cm column of hydroxylapatite equilibrated in Buffer A. After the unbound protein was washed off with two bed-volumes of Buffer A the adsorbed protein was eluted with a linear gradient between 20-150 m*M* K-phosphate containing 1.0 mM DTT, pH 7.0, in a total volume of 800 ml. Fractions of 4 ml were collected. The fractions (#137-147) in the gradient that contained the activity of shikimate dehydrogenase were pooled and concentrated by ultrafiltration on a PM-10 membrane (~10 ml). The ionic strength was reduced to that of Buffer A as follows. The concentrate was diluted 5-fold with 5 m*M* K-phosphate, pH 7.0, containing 1.0 m*M* DTT and the concentration repeated. The solution was diluted (final volume: 9 ml) with the same buffer until the concentrate had the same conductance as Buffer A.

Affinity chromatography.

The above concentrate was loaded on a 1 x 28.5 cm column of 2'-5'-ADP agarose equilibrated with Buffer A. The unbound protein was removed by washing with two bed-volumes of Buffer A and the shikimate dehydrogenase was eluted with a linear

gradient (200 ml) between buffer A and 150 m*M* K-phosphate, pH 7.0, containing 0.75 m*M* NADP and 1.0 m*M* DTT. Two peaks of activity resulted with the leading peak (SDH-L; fractions 85-87) containing most of the activity as shown in Fig. 2-11. Each peak was concentrated separately and stored as such at 4°C, or at -80°C for extended storage. The results of the entire procedure are summarized in Table 2-2.

Analysis of the Purified Preparation.

Figure 2-12A shows the results of a SDS-polyacrylamide gel electrophoretic analysis of a sample of the preparation (0.75 μg) followed by silver staining of the gel (Bio-Rad silver stain kit). The figure shows the results for SDH-L. Two bands were seen, one at ~25,000 and another at ~35,000 daltons. Given the monomeric M_r of the SDH (27,000 as determined by gel filtration), the band at M_r ~ 25,000 represents the SDH. The identity of the other band is uncertain, although it is likely that it is another of the proteins in the complex and not an unrelated contaminant. One candidate is DAHP synthase since it has a monomeric weight of M_r ~35,000. However, DAHP synthase activity could not be detected in the final preparation of shikimate dehydrogenase.

The shikimate dehydrogenase eluted from the various steps in the purification was electrophoresed on a polyacrylamide gel under non-denaturing conditions and the shikimate dehydrogenase activity visualized with an enzyme activity-

Fig. 2-11. Purification of shikimate dehydrogenase. Affinity chromatography (see text) of shikimate dehydrogenase after successive DEAE-cellulose and hydroxylapatite chromatography used in the purification scheme for the enzyme as described in the text.

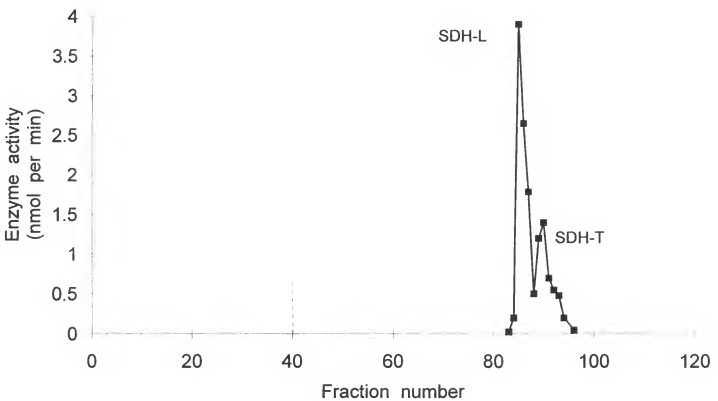

Enzyme activity
(nmol per min)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
Fraction number
SDH-L
SDH-T

Table 2-2. Results of the purification protocol for shikimate dehydrogenase.

Column matrix	Volume (ml)	Protein (mg)	Specific activity	Total activity[a]	Yield %	Fold pure
Crude extract	70	1225	-[b]	-	-	-
DEAE-cellulose	20	42	72.11	3028	100[c]	1.0
Hydroxylapatite	9	2.34	92.3	216	7.1	1.28
Affinity chromat.						
SDH-L[d]	1.8	0.0288	1570	45.20	1.5	21.77
SDH-T	1.4	0.0084	1220	10.24	0.33	17.70
combined	3.2	0.0372	1416	52.60	1.74	19.60

[a] nmol min^{-1} mg^{-1} protein
[b] not determinable due to exceedingly high background
[c] The activity of the DEAE-cellulose preparation was taken as 100% since activity in crude was not determinable.
[d] SDH-L, leading peak on affinity chromatography
SDH-T, trailing peak on affinity chromatography

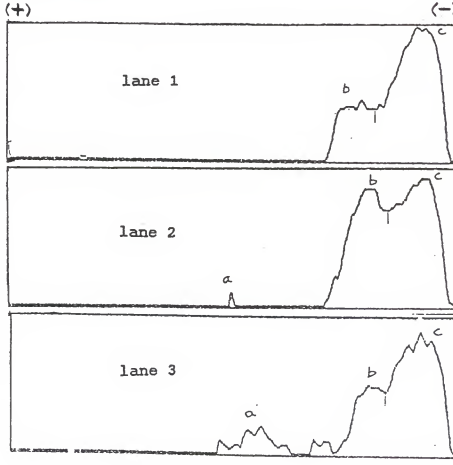
Fig. 2-12. Polyacrylamide-gel-electrophoresis analysis of the purified preparation of shikimate dehydrogenase. (A). Laemmli-SDS-PAGE of purified shikimate dehydrogenase. Samples were separated on a 12% gel formed according to the procedure of Blackshear (1984). A 0.75-μg aliquot of the purified SDH-L was loaded; the gel was silver-stained after the run using the Bio-Rad silver stain kit. *lane 1*: molecular weight markers, *lane 2*: preparation of SDH-L. (B). "Native" polyacrylamide-gel-electrophoresis of shikimate dehydrogenase. Aliquots of the enzyme preparations recovered after various steps in the purification were used. *Lane 1*: ~100 μg of the DEAE-cellulose preparation, *lane 2*: ~15 μg of the preparation eluted from hydroxylapatite, *lane 3*: 0.75 μg of the affinity-purified preparation. Gels (9.4% resolving gel, 2% stacking gel) were prepared and run according to the procedure of Orr et al. (1972) at 4°C, except that TEA-MOPS (3-[*N*-morpholino]propane sulfonic acid) was used in place of TEA-TES. Gels were stained for shikimate dehydrogenase with a solution of 0.75 mM shikimate, 0.5 mM $NADP^+$, 15 mg 3-(4:5-dimethyl-2-thiazolyl)-2:5-diphenyl-2H-tetrazolium bromide (MTT), and 1 mg phenazine methosulfate (PMS) in 50 ml 100 mM K-phosphate, pH 7.0. (C). Silver stain of the gel run in (B). (D). Video densitometer scan of the gel run in (B). The gel was scanned using a Zeineh Video Densitometer (Biomed Instruments, Fullerton, CA). Multiple scans of each lane were accumulated to optimize signal-to-noise ratio. Lanes were individually normalized by setting the darkest band in that lane as 100%.

(A).
1
2
94000
67000
43000
30000
20100

(B).

(C).

(D).



stain. The shikimate dehydrogenase appeared to be polymorphic. Figure 2-12B-D shows the results of such an experiment. At least two bands are seen for all the preparations loaded on the gel in Fig. 2-12B. In panel (B), lane 3 that contains the affinity purified enzyme preparation of SDH-L three bands were detected with the third band being barely visible. To better define the bands in the gel it was scanned with a video-densitometer and these results are presented in Fig. 2-12D. In all lanes, as expected, two peaks that are coincident in position, marked as peaks *b* and *c*, are evident. Peak *a* that is now better visible in lane 3 on the densitograph is a very small peak in lane 2, but is absent in lane 1. There appears to be a progressive increase in the size of peak *a* in going from lane 1 to lane 3. The presence of the second protein in the SDS gels of Fig. 2-12A would allow for an explanation for this polymorphism if it is assumed to form part of the complex which contains shikimate dehydrogenase. This possibility remains to be tested. Panel (C) shows the same gel when stained with the silver stain.

Indirect Evidence for the Involvement of Dehydroquinate Synthase in the Complex.

The above results prompted an investigation to address the question of whether other proteins are present in the complex. Likely candidates were the two enzymes, dehydroquinate synthase and dehydroquinate dehydratase, enzymes that lie between DAHP synthase and shikimate

dehydrogenase in the common pathway. Figure 2-13 shows the chromatographic profiles for three of these four enzymes from crude extracts run on a DEAE-cellulose column according to the conditions depicted in Fig. 2-6. Dehydroquinate synthase appears to be coincident with the shikimate dehydrogenase. These enzyme fractions were pooled. This pool contained dehydroquinate dehydratase activity (33.33 nmoles product formed per min).

The pool was concentrated by ultrafiltration on a PM-10 membrane and loaded on a 1 x 23 cm column of 2':5'-ADP agarose equilibrated with Buffer A. The column was washed with two bed-volumes of Buffer A and then with Buffer B containing 0.5 m*M* NADP. The column fractions (2.2 ml each) were assayed for the enzymes dehydroquinate synthase, shikimate dehydrogenase and dehydroquinate dehydratase. The resulting activity profiles are shown in Fig. 2-14. The dehydroquinate synthase eluted coincidentally with the shikimate dehydrogenase that binds the affinity column (Fig. 2-14). This result is consistent with the idea that dehydroquinate synthase is a part of the complex involving shikimate dehydrogenase.

This was indirectly verified in a separate experiment as follows. Figure 2-15 shows the elution profile for dehydroquinate synthase from strain 2013 after chromatography on hydroxylapatite under the conditions depicted in Fig. 2-1. The peak fractions of shikimate dehydrogenase and dehydroquinate synthase were separated. The fractions

Fig. 2-13. DEAE-cellulose chromatography of crude extracts of strain 2013. The column was run as described for Fig. 2-6. The activity of dehydroquinate synthase (DHQS), assayed by the coupled assay under 'Materials and Methods', is reported as A_{549}; the value truly represents the calculated decrease in A_{549}, i.e. a value of 1.0 represents a $-\Delta A_{549}$ of 1.0.

Absorbance at 280 nm/549 nm
SDH activity (nmol per min)
Fraction number
Protein
DAHPS
DHQS
SDH
0
0.5
1
1.5
2
2.5
3
3.5
0
20
40
60
80
100
120
140
160
180

Fig. 2-14. Affinity chromatography on 2'-5'-ADP agarose of the enzymes eluted from DEAE-cellulose as depicted in Fig. 2-13 The procedures have been described in the text. Dehydroquinate dehydratase (DQT), shown here, was not assayed off the column in Fig. 2-13, but was detected in the pooled concentrate from the same column. Dehydroquinate synthase activity is reported as described in Fig. 2-13.

Absorbance at 549 nm (DHQS)
DQT Activity (nmol per min)
SDH activity (nmol per min)
Fraction number
DQT
DHQS
SDH
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
1
2
3
4
0
20
40
60
80
100
120
140
160
180

Fig. 2-15. Hydroxylapatite chromatography of crude extracts of strain 2013. The column was run as described in the section on 'Materials and Methods'. The figure shows the elution profiles of dehydroquinate synthase and shikimate dehydrogenase. Dehydroquinate synthase activity is reported as described for Fig. 2-13.

Absorbance at
280 nm / 549 nm
SDH activity
(nmol per min)
Fraction number
DHQS
Protein
SDH
90 mM KP
110 mM KP
V
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0.5
1.5
2.5
3
20
40
60
80
100
120
140
160
180

containing the dehydroquinate synthase activity, that were substantially free from shikimate dehydrogenase activity, were pooled and concentrated on a PM-10 membrane. The concentrate was then chromatographed on the affinity column. Dehydroquinate synthase eluted in the wash from the affinity column (data not shown) showing that it was eluted in the gradient of Fig. 2-14 by virtue of its association with shikimate dehydrogenase.

In Fig. 2-14 the enzyme dehydroquinate dehydratase is seen in the wash fractions and evidence for its involvement in the complex is sketchy.

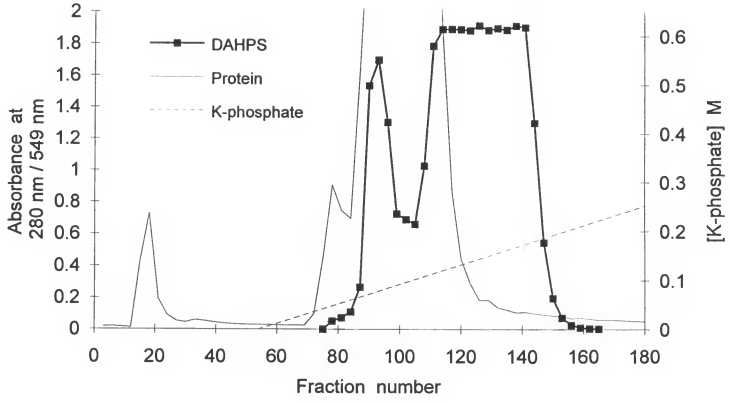
Regulation of the Aromatic Pathway at the Level of DAHP Synthase and Shikimate Dehydrogenase.

DAHP synthase in N. gonorrhoeae is regulated both by feedback inhibition and repression (see below). The activity of DAHP synthase is inhibited by phenylalanine, though only to a maximum of ~70% (Fig. 2-2).

In another class of mutants selected for pfP-resistance, 2013-8, the repression control is affected leading to a 30-50-fold overexpression of the DAHP synthase (Fig. 2-16).

The shikimate dehydrogenase (SDH-L as separated in the purification scheme for the shikimate dehydrogenase) was found to be sensitive to inhibition by chorismate, up to 40% by 1 m*M* chorismate. Phenylalanine, tyrosine, tryptophan and prephenate at 1.0m*M* had no effect on the activity. A kinetic

Fig. 2-16. Chromatography of strain 2013-8, a pFP-resistant mutant. Crude extracts of strain 2013-8 were prepared from cells grown in the presence of 0.1 mM pFP, and chromatographed essentially as in Fig. 2-1. Only DAHPS activity is reported (A_{549}).


DAHPS
Protein
K-phosphate
Absorbance at 280 nm / 549 nm
[K-phosphate] M
Fraction number
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0.1
0.3
0.5
20
40
60
80
100
120
140
160
180

analysis of the inhibition (Fig. 2-17) shows it to be a mixed-type inhibition.

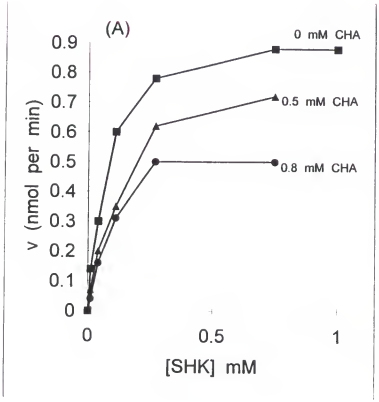
The DS-Phe isozyme in the β-subdivision of Purple Bacteria.

N. gonorrhoeae belongs to the β-subdivision of purple bacteria (Woese, 1987). Within the β-subdivision, studies with three other bacteria [*Alcaligenes eutrophus* (Friedrich and Schlegel, 1975) *Pseudomonas acidovorans* (Berry et al., 1985) and *Alcaligenes faecalis* (Subramaniam et al., manuscript submitted)] have revealed two chromatographic forms of the DS-Phe isozyme. In the case of *P. acidovorans* the two forms were found to be interconvertible. Selected other bacteria within the subdivision as representatives of major lineages were examined to see if the two forms of the DS-Phe isozyme were widely distributed or not. These were *Pseudomonas testosteroni* (ATCC 17409), *Pseudomonas pickettii* (ATCC 27511) and *Nitrosomonas europaea* (Schmidt strain).

Chromatography of crude extracts from *P. testosteroni* on DEAE-cellulose resolved DS-Phe and DS-Tyr isozymes as shown in Fig. 2-18. Two forms of the DS-Phe isozyme were seen. Like *P. acidovorans*, a DS-Tyr isozyme is seen in conjunction with the two DS-Phe species (see Table 2-3). In *N. gonorrhoeae* and *A. faecalis* (Subramaniam et al., manuscript submitted), only DS-Phe has been observed.

Thus, the occurrence of two forms of DS-Phe appears to be common to many other bacteria in the β-subdivision as

Fig. 2-17. Kinetics of inhibition of SDH by chorismate. For all assays the reactions were initiated by adding substrate, shikimate, at indicated concentrations, to a reaction mixture containing 0.5 mM $NADP^+$, inhibitor as the case may be, and enzyme pre-warmed to 37°C, in Buffer A; final volume was 400 μl. **(A)**. Substrate saturation curves for SDH at fixed concentrations of chorismate (CHA). **(B)**. Double-reciprocal plots for data presented in (A).


(A)
0 mM CHA
0.5 mM CHA
0.8 mM CHA
v (nmol per min)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
[SHK] mM

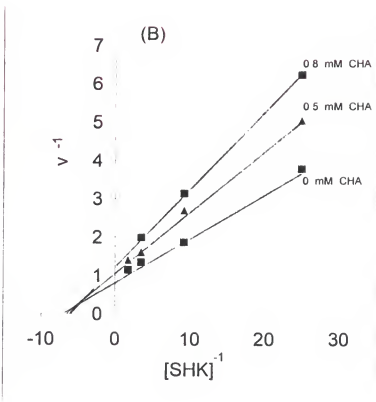

(B)
0 8 mM CHA
0 5 mM CHA
0 mM CHA
v -1
7
6
5
4
3
2
1
0
-10
0
10
20
30
[SHK]-1

Fig. 2-18. Activity profiles of enzymes from *P. testosteroni* ATCC 17409 on DEAE-cellulose. Crude extract (100 mg protein) was loaded on a 1.5 x 20 cm DEAE-cellulose column in Buffer A. After washing the column with two volumes of equilibration buffer, bound protein was eluted by application of a linear gradient between 0-400 m*M* KCl in Buffer A in a total volume of 350 ml. The dashed diagonal line follows the progression of the gradient. DAHP synthase activity is reported as A_{549}. Elution of protein was monitored as A_{280}.

Absorbance at
280 nm / 549 nm
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
180
Fraction number
[KCl] M
0.6
0.5
0.4
0.3
0.2
0.1
0
DS-Phe
DS-Tyr

Table 2-3. Distribution of DAHP synthase isozymes within organisms of the β-subdivision.

Organism[a]	Number of DS-Phe isozymes	Other isozymes (number)	Reference
Subgroup β-1			
P. testosteroni	2	DS-Tyr (1)	This study
P. acidovorans	2	DS-Tyr (1)	(1)
Subgroup β-2			
P. pickettii	1	DS-Tyr (1)	This study
A. eutrophus	2	None [b]	(2)
A. faecalis	2	None	(3)
Subgroup β-3			
N. gonorrhoeae	2	None	This study

[a]Classification is from Woese, 1987.
[b]Phenylalanine and tyrosine inhibit cummulatively. Individually phenylalanine inhibits to 60% and tyosine to 20% at 0.5 mM.
Refs: (1). Berry et al., 1985 (2). Friedrich and Schlegel, 1975 (3). Subramaniam et al., manuscript submitted.

summarized in Table 2-3 . This suggests that DS-Phe might exist as part of a dissociable complex, in a phylogenetic cluster of organisma which includes *N. gonorrhoeae*.

P. pickettii expressed single DS-Phe and DS-Tyr isozymes (data not shown). In the case of *N. europaea* no information on DAHP synthase could be gathered due to technical difficulties.

CHAPTER 3
THE OVERLAPPING SUBSTRATE-UTILIZATION CAPABILITIES OF DAHP SYNTHASE AND KDOP SYNTHASE

Introduction

As shown in the previous chapter, in the chromatographic profile (on hydroxylapatite) of the Aro$^-$ strain, shown in Fig. 2-3, a minor activity of DAHP synthase was detected. This activity was unregulated by the aromatic amino acids. This property is characteristic of the unregulated DAHP synthase isozyme, DS-0 (Ahmad et al., 1986). In bacteria, the DS-0 species had hitherto been recognized only in *Oceanospirillum* and *Acinetobacter* species (Ahmad et al., 1986). It was possible that *N. gonorrhoeae* expressed this isozyme at a low level.

Another option followed from a recent study from our laboratory (Doong et al., 1991). It was found that the substrate specificity of the enzyme, 3-deoxy-D-*manno*-octulosonate-8-phosphate synthase (KDOP synthase), from potato was sufficiently relaxed for it to masquerade as a DAHP synthase, a consequence of its ability to utilize E4P as a substrate. Indeed, this ambiguity led to the recognition of the presence of KDOP synthase for the first time in a plant system when the product of its activity with its favored

substrate, arabinose-5-phosphate (A5P), was confirmed to be KDOP (Doong et al., 1991).

3-Deoxy-D-*arabino*-heptulosonic acid-7-phosphate (DAHP) and 3-deoxy-D-*manno*-octulosonic acid-8-phosphate (KDOP) are analogous 7- and 8-carbon 2-keto-3-deoxy sugars that are synthesised by enzymes which are members of two functionally unrelated pathways. DAHP synthase forms DAHP as the acyclic precursor of the aromatic amino acids in bacteria, lower eukaryotes and plants (Bentley, 1990), while KDOP synthase forms KDOP as a critical component of the lipopolysaccharide of gram-negative bacteria (Mayer et al., 1989). Both enzymes catalyze an overall aldol-type condensation of phosphoenoyl pyruvate (PEP) with an aldose; erythrose-4-phosphate (E4P) in the case of DAHP synthase, and arabinose-5-phosphate (A5P) in the case of KDOP synthase. If A5P is viewed as the homologue of E4P, the reactions are strikingly similar. This similarity is reflected at the level of mechanistic detail.

These two enzymes, along with enolpyruvoylshikimate 3-phosphate synthase and UDP-*N*-acetylglucosamine enolpyruvoyl transferase, describe a small class of PEP-utilizing enzymes that catalyze the C-O-bond cleavage in releasing Pi from PEP (Hedstrom and Abeles, 1988; Anderson and Johnson, 1990). This contrasts with the familiar nucleophilic attack at the phosphorous atom of PEP that results in P-O-bond cleavage by the action of enzymes like pyruvate kinase (Harrison et al.,

1955), phosphoenolpyruvate carboxylase (Maruyama et al., 1966) and phosphoenolpyruvate carboxykinase (Chang et al., 1966).

In the present case a distinction between the two activities was made by examining the product of the reaction of the KDOP synthase with E4P. The sterochemistry of the product has been investigated with the periodate-oxidation-TBA assay and these results allow some speculation on the stereospecific course of the reaction in comparison with the reaction of DAHP synthase.

Results and Discussion.

Relaxed Substrate Specificity of the KDOP Synthase Reaction.

Fig. 3-1 shows the activity profile for DAHP synthase and KDOP synthase after fractionation of crude extracts of *N. gonorrhoeae* VHC 3102 on hydroxyapatite. When the column fractions were assayed for the activity of KDOP synthase, the low activity hump around fractions 100-110 was found to be overlapping with the profile of KDOP synthase, suggesting that this apparent DAHP synthase activity may be attributed to a substrate ambiguity of KDOP synthase, especially since a precedent had been established (Doong and Jensen, 1992). The effect of increasing concentrations of E4P relative to A5P at a fixed concentration of PEP on the product yield using this preparation of enzyme is presented in Fig. 3-2. The amount of the product formed using E4P is maximized at 3mM of E4P and is

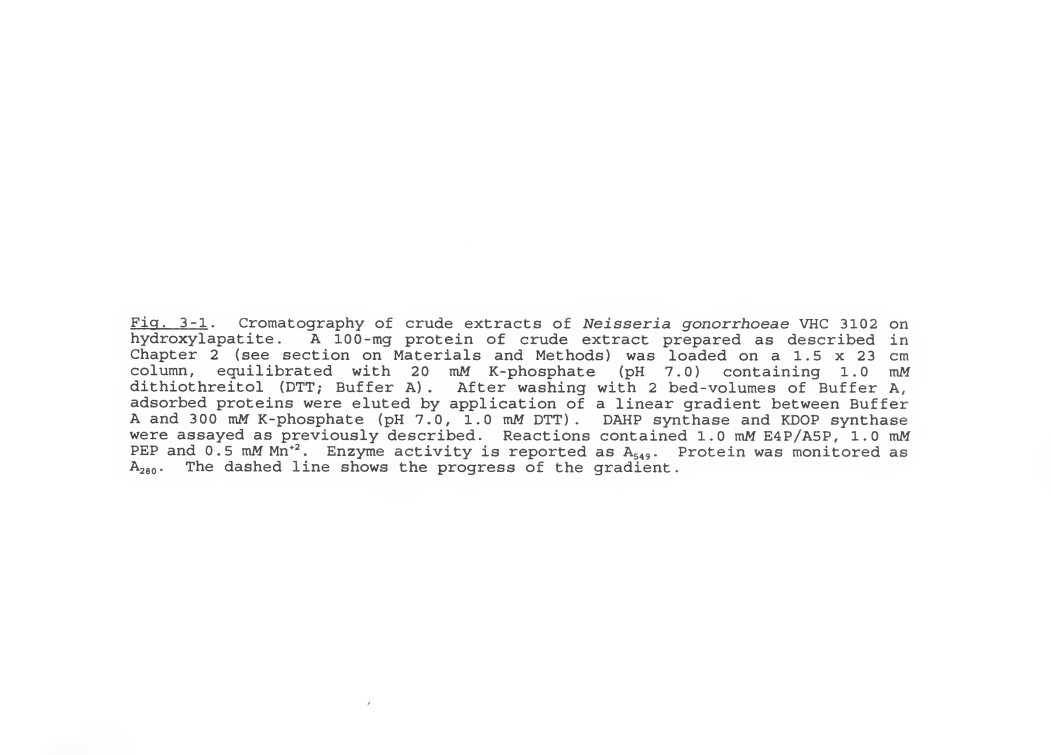


Fig. 3-1. Cromatography of crude extracts of *Neisseria gonorrhoeae* VHC 3102 on hydroxylapatite. A 100-mg protein of crude extract prepared as described in Chapter 2 (see section on Materials and Methods) was loaded on a 1.5 x 23 cm column, equilibrated with 20 m*M* K-phosphate (pH 7.0) containing 1.0 m*M* dithiothreitol (DTT; Buffer A). After washing with 2 bed-volumes of Buffer A, adsorbed proteins were eluted by application of a linear gradient between Buffer A and 300 m*M* K-phosphate (pH 7.0, 1.0 m*M* DTT). DAHP synthase and KDOP synthase were assayed as previously described. Reactions contained 1.0 m*M* E4P/A5P, 1.0 m*M* PEP and 0.5 m*M* Mn^{+2}. Enzyme activity is reported as A_{549}. Protein was monitored as A_{280}. The dashed line shows the progress of the gradient.

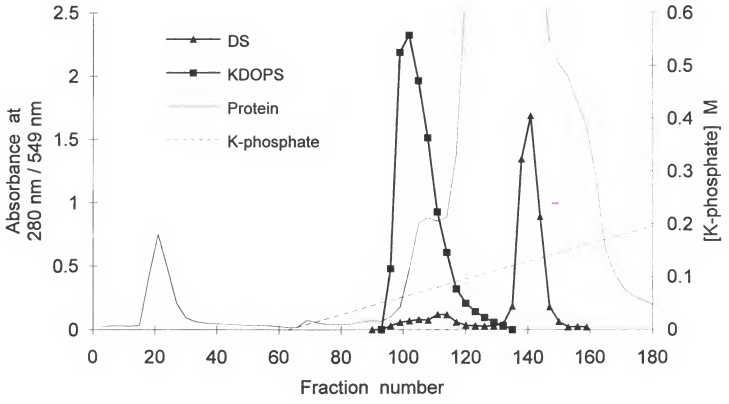
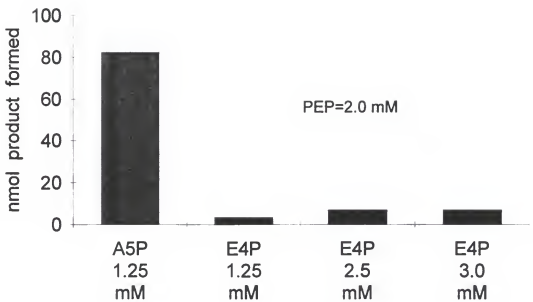

DS
KDOPS
Protein
K-phosphate
Absorbance at 280 nm / 549 nm
[K-phosphate] M
Fraction number
0
0.5
1
1.5
2
2.5
0.1
0.2
0.3
0.4
0.5
0.6
20
40
60
80
100
120
140
160
180

Fig. 3-2. The relative ability of KDOP synthase to use E4P and A5P at a fixed concentration of PEP. Equal aliquots of partially-purified KDOP synthase as in Fig. 3-1 were incubated with the appropriate concentrations of E4P and A5P in Buffer A (see Fig. 3-1) with 2 m*M* PEP, and 1.0 m*M* Mn^{+2}. The nmol of product formed plotted on the ordinate scale were arrived at using an ϵ value of 1.03×10^4 M^{-1} cm^{-1} for the KDOP-derived chromophore and assuming an ϵ value equal to that for DAHP (5.0×10^4 M^{-1} cm^{-1}) for the E4P derived product.


nmol product formed
100
80
60
40
20
0
PEP=2.0 mM
A5P 1.25 mM
E4P 1.25 mM
E4P 2.5 mM
E4P 3.0 mM

equal to 7.88 nmoles of DAHP equivalents at a fixed concentration of 2 mM PEP. One equivalent of DAHP represents that concentration of DAHP that would give the observed absorbance value when the ϵ value for DAHP is used. The standard oxidation time of 30 minutes of the DAHP synthase assay was used. No activity was seen with D- or L-glyceraldehyde-3-phosphate (1.5 mM). However, D-ribose-5-phosphate (3 mM), an isomer of A5P, was utilized to the same extent as E4P. To distinguish an overlapping DAHP synthase of low activity at the position of the KDOP synthase the product of the reaction with E4P was examined by periodate-oxidation. As described in the next section, the product behaves quite differently from DAHP.

The Stereochemistry of the Resultant C_7 Product.

Periodate oxidation coupled with the thiobarbituric acid (TBA) assay for 2-keto-3-deoxy-sugars provides a simple method to examine the relative configuration of the hydroxyl groups at C4 and C5 in sugars like DAHP and KDOP. The rate of chromophore formation is directly proportionate to the ease of oxidation at C4-C5 (Ghalambor et al., 1966); all 2-keto-3-deoxy-sugars giving upon C4-C5 cleavage the same TBA-reactive ß-formylpyruvate, the rate of release of this reactive intermediate being determined by the relative ease of oxidation at C4-C5 by periodate.

Thus, KDOP is rapidly oxidized by periodate as a consequence of the *cis* orientation of the C4 and C5 hydroxyl groups (Ghalambor et al., 1966), in contrast to the much slower oxidation of the *trans* configuration of these hydroxyls seen in DAHP. The orientation of the C4-hydroxyl, in particular, in KDOP and DAHP is dictated by the stereochemistry of the addition across the face of the carbonyl group in the substrate sugar. In the case of both KDOP synthase and DAHP synthase, the orientation of the hydroxyl generated by the addition of PEP is such that C4 has a (R)- configuration in the products KDOP and DAHP.

In order to accumulate sufficient amounts of the E4P/KDOP synthase derived C_7 product, the reaction was scaled up as follows. A 1-ml reaction mixture containing 3.6 mM E4P, 2 mM PEP, 1 mM dithiothreitol and 0.5 mM Mn^{+2}, and 375 μg of partially purified KDOP synthase in 75 mM potassium phosphate buffer (pH 7.0) was incubated at 37°C for 5 hrs. The reaction mixture was then transferred to 4°C and stored overnight. A 40 μl amount of 100% (w/v) trichloroacetic acid was added, mixed and the mixture kept on ice for 5 min. The precipitated protein was removed by centrifugation and the supernatant diluted two-fold with buffer and used for oxidation studies. DAHP was accumulated in a similar 1-ml reaction mixture except that it contained 1 mM E4P, 1 mM PEP, and 940 μg crude extract preparation of *Ps. testosteroni* (ATCC 17409) incubated at 37°C for 20 min; the supernatant was diluted two-fold with buffer

before use. A 100-μl sample of each supernatant was used. A notably large amount of the C_7 product was formed corresponding to a concentration of 0.35 mM of DAHP equivalents.

Fig. 3-3 shows a comparison of the results of the kinetics of periodate-oxidation of the C_7-X product and DAHP. It is evident that the C_7-X product is very rapidly oxidized in contrast to DAHP, with the oxidation being complete within 4 min of incubation, and almost 65% of chromophore released within 1 min of the oxidation. (cf. 17% for DAHP). These results exactly parallel those of Doong *et al.* (1991) who compared the rates of periodate-oxidation of KDOP and DAHP under identical conditions. The C_7-X product behaves like KDOP and authentic KDO which are maximally oxidised within 5 min of incubation. Thus the relative configuration of the hydroxyls at C4-C5 in the C_7-X product is most likely *cis* and this isomer is the predominant product, i.e. the 4(S)-isomer is formed *versus* the 4(R)-KDOP and 4(R)-DAHP. The product is, thus, postulated to be the D-*ribo*-analogue of DAHP viz. 3-deoxy-D-*ribo*-heptulosonic acid 7-phosphate.

The C_7-X product was tested as a substrate for dehydroquinate synthase, the enzyme that uses DAHP as a substrate to form dehydroquinate. Fig. 3-4 shows the elution profile of dehydroquinate synthase from hydroxylapatite, as described in the previous chapter. The enzyme was assayed by

Fig. 3-3. Kinetics of periodate oxidation of the enzymatic products of KDOP synthase and DAHP synthase reactions with E4P and PEP. Products were accumulated as described in the text, and a 100-μl sample was used in the periodate-oxidation-TBA assay procedure used for the determination of DAHP, except that the oxidation was terminated at the appropriate time intervals. Maximal absorbance at 549 nm in each case was assigned a relative value of 100% and corresponded to 0.721 for DAHP and 1.482 for the E4P/KDOP synthase derived product (C_7-X).

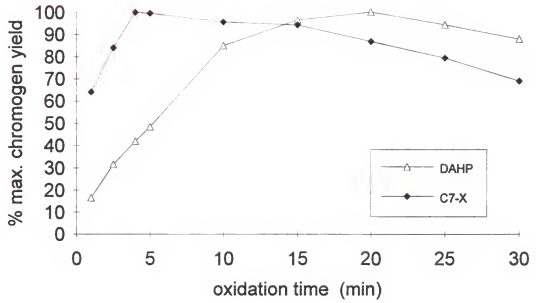
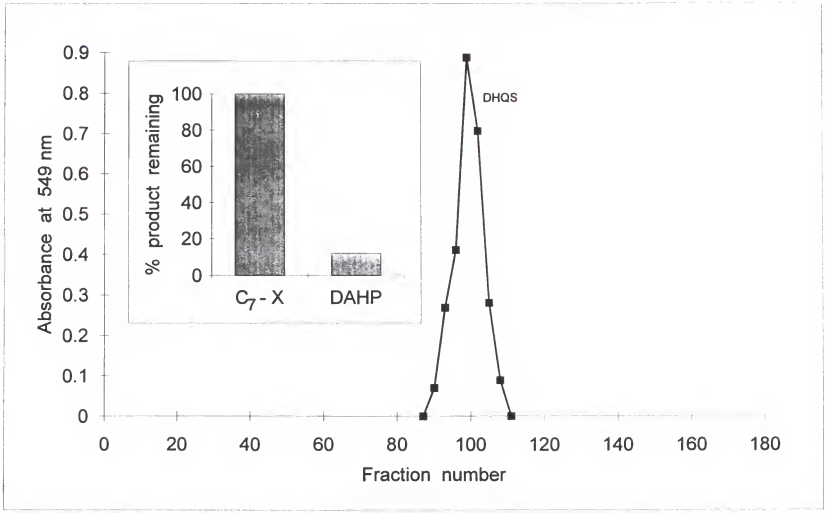

% max. chromogen yield
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
oxidation time (min)
DAHP
C7-X

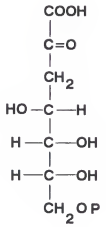
Fig. 3-4. The reaction of C_7-X with dehydroquinate synthase. Dehydroquinate synthase activity is profiled as eluted off the column in Fig. 3-1. Enzyme activity is reported for simplicity as A_{549}. Note that the values actually represent ΔA_{549} where a value of 1.0 corresponds to 48.0 nmol of DAHP consumed. Insert shows the % product remaining after incubation of C_7-X and DAHP with dehydroquinate synthase as described in the text. A value of 100% corresponds to 0.24 m*M* DAHP and 0.1m*M* of DAHP equivalents of C_7-X.


Absorbance at 549 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
180
Fraction number
DHQS
% product remaining
100
80
60
40
20
0
C_7 - X
DAHP

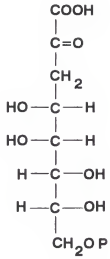
following the disappearance of DAHP, using the TBA-assay. The C_7-X product accumulated as described above was incubated with the dehydroquinate synthase for 20 min in a reaction mixture containing 0.5 mM Co^{+2}, and 50 μM NAD^+. The C_7 product was not utilized as substrate (Fig. 3-4, insert). As a control, the enzyme was incubated with authentic DAHP accumulated above (Fig. 3-4, insert). Thus the C_7-X product is not a substrate for dehydroquinate synthase consistent with its likely identity as 3-deoxy-D-*ribo*-heptulosonic acid 7-phosphate. Two conclusions follow from this result. The low level of apparent DAHP synthase activity does not in fact arise from a protein having the ability to catalyze the DAHP synthase reaction since the KDOP synthase does not make DAHP as a product.

At first glance, the latter product is not the one expected to form in the KDOP synthase reaction in consideration of similarity of the the mechanism of its reaction with that of the well-studied DAHP synthase (DeLeo et al., 1973; Ganem, 1978). The condensation of E4P and PEP by DAHP synthase proceeds by a stereospecific addition across the *si* face of C3 of PEP (of a (Z)-[3-^{3}H]-PEP derivative) and the *re* face of C1 of E4P (Floss et al., 1972). The resulting DAHP has a (R)-configuration at C4 (Fig. 3-5 and Fig. 3-6) with the newly formed hydroxyl being *trans* to the the one on C5. In the case of KDOP synthase reaction, based on the established structure of KDOP, the same stereochemistry, 4(R)-, at C4 is

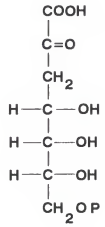
Fig. 3-5. Chemical structures of DAHP, KDOP and DRHP.



3-Deoxy-D-*arabino*-
heptulosonate 7-phosphate
(DAHP)

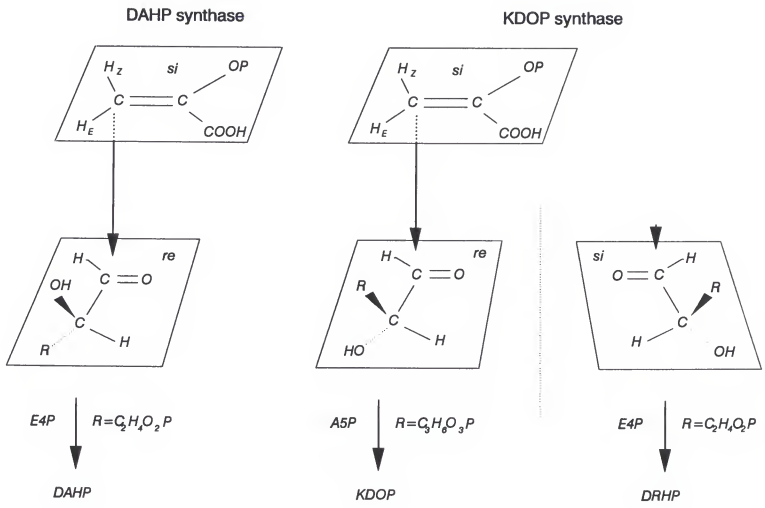


3-Deoxy-D- *manno*-
octulosonate 8-phosphate
(KDOP)



3-Deoxy-D- *ribo*-
heptulosonate 7-phosphate
(DRHP)

Fig. 3-6. Representation of the mechanism of formation of DAHP by DAHP synthase, KDOP by KDOP synthase and DRHP by KDOP synthase. The mechanism for the formation of DRHP is hypothetical. In the case of the formation of DRHP it is assumed that the addition is across the *si* face of PEP by analogy.


DAHP synthase
KDOP synthase
H z
si
OP
C
C
H E
COOH
re
H
C
O
OH
C
R
H
E4P
R=C2H4O2P
DAHP
A5P
R=C3H6O3P
KDOP
HO
si
O
R
E4P
R=C2H4O2P
DRHP

generated and the reaction most likely proceeds with the same stereospecificity as the DAHP synthase reaction. The configuration of the substrate (A5P) disposes the C5 *cis* to the newly formed hydroxyl at C4. To form the 4(S)-isomer, as in the case of 3-deoxy-D-*ribo*-hepulosonic acid, the addition should proceed across the *si* face of C3 of PEP and the *si* face of C1 of E4P as opposed to the *re* face of the carbonyl in arabinose-5-phosphate. E4P is presumably accomodated at the active site such that the C1-carbonyl presents the face opposite to that across which PEP adds in E4P in the DAHP synthase reaction and in A5P in the "true" KDOP synthase reaction. I have, of course, assumed that the *si*-face of PEP is consistently involved in all these reactions, since this appears to prevail in other reactions involving PEP, including those that do not involve C-O bond cleavage (Rose et al., 1969). The D-*ribo*-product is apparently the predominant product. It follows that the reaction is not mediated by a low-activity DAHP synthase, and the KDOP synthase in reality does not make DAHP when utilising E4P. One could predict that (at least in this case) the KDOP synthase cannot function *in vivo* as a DAHP synthase, if for example, a KDOP synthase gene placed in a high copy-number plasmid were used to complement a DAHP synthase defect.

Substrate-ambiguity and Reversal of Stereospecificity as More Prevalent Characteristics of DAHP- and KDOP Synthases.

These results are not surprising in light of the earlier reports from our laboratory regarding these phenomenona as seen with both the DAHP synthase and KDOP synthase of higher plants. Essentially identical results were seen with the KDOP synthase of *Spinacia oleracea* (Doong et al., 1992) which showed a better ability to use E4P (24% as well as A5P) than the *N. gonorrhoeae* KDOP synthase. The resultant 7-carbon product was the D-*ribo*-derivative as revealed by periodate-oxidation studies, and the reaction with E4P presumably proceeding with the opposite stereochemistry as elaborated here. More striking, however, is the extreme substrate ambiguity of one of the two DAHP synthase isozymes, DS-Co, which is able to catalyze condensations between PEP and a bewildering array of aldoses that include the phospho- and dephospho-counterparts of glycoaldehyde, glyceraldehyde, erythrose, threose, arabinose, ribose and glucose (Doong et al., 1992). That is probably the only report of a DAHP synthase that shows latitude in substrate specificity. Indeed, the efficiency of catalysis, as defined by the V_{max}/K_m ratio, with glycoaldehyde exceeded that with E4P, while that of glyceraldehyde-3-phosphate equaled that with E4P. When the specific products are subjected to periodate-oxidation, the product, for example, with glyceraldehyde-3-phosphate appears to have a stereochemistry opposite (*cis*-C4-C5-hydroxyls) to

that expected from the condensation of E4P (*trans*-C4-C5), a reaction that is as efficient as that with E4P.

A question of interest, then, is to see if this ability to utilize alternative substrates is consistent with a relaxed stereospecificity of the reaction in those KDOP/DAHP synthases that show this substrate ambiguity. Although, I use the term "relaxed stereospecificity" the reaction *per se* with E4P (of the KDOP synthase from *N. gonorrhoeae*) is presumably stereospecific in generating the D-*ribo*-analogue. Since KDOP synthase and DAHP synthase are thought to have evolved from a common ancestor this degree of non-specificity may reflect a residual characteristic carried-forward from the common ancestor. Such a scenario would be consistent with the hypothesis of Jensen (1976) where substrate specificity in analogous enzyme reactions is postulated to have arisen from a common ancestor of broad specificity in the recruitment of pre-existing enzymes in the evolution of new function.

2-Keto-3-deoxy-sugars are common in the cell wall, lipopolysaccharide, and extracellular polysaccharides of bacteria (Mayer et al., 1989), but their biosynthetic routes are not known. It would be logical to envisage their synthesis by reactions analogous to those of DAHP synthase and KDOP synthase. From an evolutionary standpoint, given the existence of the broad catalytic ability to condense PEP and aldoses, selection for specific function could occur at two levels: one, at the level of the stereospecificity of the

reaction which has a four-fold freedom, and the other, at the level of specificity for the appropriate sugar-substrates. Indeed, Bauerle (personal communication) has shown that the three isoenzymes of DAHP synthases and KDOP sythase in *E. coli* share at the level of amino acid sequence several conserved regions, two of which are directly involved in substrate binding. Thus, it would not be surprising if enzymes form other sources show varying degrees of substrate-specificities and relaxed stereochemistries that reflect divergence from a common ancestor.

CHAPTER 4
CONCLUSIONS

Data presented here expand knowledge on the biosynthesis of aromatic amino acids in *N. gonorrhoeae*.

In *N. gonorrhoeae* two forms of DAHP synthase-Phe were initially demonstrated by hydroxylapatite chromatography. No other isozyme of DAHP synthase was seen, and the two forms were similar in their response to the inhibitor, phenylalanine. Further experiments showed that these two forms arose from the dissociation of a multifunctional complex involving DAHP synthase-Phe and shikimate dehydrogenase. This complex is disrupted in natural Aro^- mutants that lack an active shikimate dehydrogenase. The loss of shikimate dehydrogenase results in a single form of DAHP synthase-Phe on hydroxylapatite chromatography.

The monomeric forms of DAHP synthase and shikimate dehydrogenase are 35,000 daltons and 27,000 daltons in size, respectively. The two enzymes appear to be components of a multimeric aggregate that has a size of ~150,000-200,000 daltons as determined by gel-filtration. PEP is able to maintain the DAHP synthase as a dimer. Moreover, PEP was found to affect the composition and size of the multimeric aggregate when monitored by these two activities.

Results from affinity chromatography, suggest that dehydroquinate synthase, the enzyme that follows DAHP synthase, is capable of association with shikimate dehydrogenase. It is likely that the above aggregate represents a multienzyme complex consisting of, in part, these three activities. It is not known if other enzymes are involved.

In one of several analog-resistant mutants, selected for resistance to growth inhibition of the phenylalanine analogs m-fluorophenylalanine and p-fluorophenylalanine, DAHP synthase activity is shown to be at least 50-fold derepressed which is indicative of regulation at the gene level. In another mutant DAHP synthase was found to be insensitive to inhibition by phenylalanine. Both forms of the enzyme that were resolved by hydroxylapatite chromatography were simultaneously desensitized as would be expected when both forms arise from the same gene product.

The presence of a complex involving DAHP synthase and shikimate dehydrogenase in *N. gonorrhoeae* is an extension of the occurrence of DAHP synthase in multifunctional complexes within the aromatic-pathway. In the two bacteria *Bacillus subtilis* and *Brevibacterium flavum* where this has been demonstrated DAHP synthase exists as one domain of a bifunctional polypeptide with chorismate mutase. In *N. gonorrhoeae*, the shikimate dehydrogenase and DAHP synthase are separate proteins. However, the association of DAHP synthase

with shikimate dehydrogenase represents the first report of the involvement of these two enzymes.

An apparent minor activity for DAHP synthase that was distinct from the activity of the DS-Phe enzyme was detected in the Aro$^-$ strain VHC 3102. This activity was shown to arise from the broad substrate-specificity of the enzyme KDOP synthase. KDOP synthase catalyzes a reaction very similar to that of DAHP synthase, between arabinose 5-phosphate and PEP and is similar in its mechanism of reaction. However, periodate-oxidation studies of the product formed from the KDOP synthase-E4P reaction suggest that the product is 3-deoxy-D-*ribo*-heptulosonate 7-phosphate and not DAHP.

Perspectives and Future Directions.

I have provided evidence for the existence of a multifunctional complex in the early portion of the shikimate pathway of *N. gonorrhoeae*. However, conclusive proof rests with demonstrating the reconstitution of the aggregate from its units.

Reconstitution of the Multifunctional Complex.

Conditions for the separation of the three enzymes DAHP synthase, shikimate dehydrogenase and dehydroquinate synthase are available. The ability of the three enzymes to complex could be tested by gel-filtration. Simple mixing of the separated enzymes followed by gel-filtration on Sephadex G-100

showed that the DAHP synthase co-eluted with the dehydroquinate synthase, but the shikimate dehydrogenase eluted at its monomeric position. Apparently other conditions are required to promote complexation. What is the role of small molecules in promoting complexation? An obvious candidate is PEP that is a known modulator of the native state of several enzymes for which it is a substrate, and promotes in this case the dimerization of DAHP synthase.

It is also possible that the enzymes associate differentially. Selective conditions may exist that promote the complexation of DAHP synthase with either shikimate dehydrogenase or dehydroquinate synthase. Again, small molecules could determine the end-result. These effects could be revealed through gel-filtration experiments in the presence and absence of phenylalanine, PEP or NADP/NADPH. Since a single DAHP synthase that is inhibited by phenylalanine controls the biosynthesis of all three amino acids, it may be that tyrosine and tryptophan may affect such a differential association. Differential association may be indicative of a mechanism of regulation where apparent allosteric control is not manifest.

An equally pertinent question is whether other enzymes are involved. Should reconstitution experiments be inconclusive it may be that one or more other enzymes/proteins are required to partake in the complex. This may be determined under the conditions of the experiments described

by Fig. 3-7 and Fig. 3-8. The high molecular weight peak *a* in each case may comprise other enzymes. Other enzymes of the pathway could be assayed for at this position. In this regard a good candidate is dehydroquinate dehydratase, the enzyme that follows dehydroquinate synthase; this would complete a "block" that would constitute the first four enzymes of the pathway. A positive result would throw much light on the results of reconstitution experiments and resolve the above two suggestions. A missing enzyme may be the key to holding the complex together.

Are the Two Forms of DS-Phe Prevalent in Superfamily A a Reflection of the Above Complex?

As mentioned earlier two forms of DS-Phe appear to be common to many bacteria within Superfamily A. In particular, the two forms of DS-Phe co-exist in some cases with a DS-Tyr isozyme. Why have two forms of the same isozyme when another isozyme is also expressed? It would be of immediate interest to see if in any of the appropriate bacteria the DS-Phe co-elutes with shikimate dehydrogenase, or other enzymes. A good choice is *P. acidovorans* ATCC 11299a where the relationship between the two forms has been examined in detail, and evidence is strong that the two forms represent the same gene-product. A positive result would lend very good support for the presence of a complex, since parallel results within other bacteria would only mean that the complex represents a conserved characteristic.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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